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**STUDIES ON THE UNFOLDING AND REFOLDING  
OF OLIGOMERIC PROTEINS**

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## ***Abbreviations***

<b>A</b>	<b>Absorbance</b>
<b>ADP</b>	<b>Adenosine diphosphate</b>
<b>AK-HDH</b>	<b>Aspartokinase-homoserine dehydrogenase</b>
<b>ANS</b>	<b>8-Anilino-1-naphthalene sulphonic acid</b>
<b>ATP</b>	<b>Adenosine triphosphate</b>
<b>BSA</b>	<b>Bovine serum albumin</b>
<b>BPTI</b>	<b>Bovine pancreatic trypsin inhibitor</b>
<b>CSA</b>	<b>Camphorsulphonic acid</b>
<b>c.d.</b>	<b>Circular dichroism</b>
<b>Cpn</b>	<b>Chaperonin</b>
<b>DNase</b>	<b>Deoxyribonuclease</b>
<b>DHFR</b>	<b>Dihydrofolate reductase</b>
<b>DTT</b>	<b>Dithiothreitol</b>
<b>EDTA</b>	<b>Ethylene diamine tetra acetate</b>
<b>ER</b>	<b>Endoplasmic reticulum</b>
<b>FUD</b>	<b>Folding unpaired domains</b>
<b>GAPDH</b>	<b>D-Glyceraldehyde 3-phosphate dehydrogenase</b>
<b>GdnHCl</b>	<b>Guanidinium chloride</b>
<b>HPLC</b>	<b>High pressure liquid chromatography</b>

ICDH	Isocitrate dehydrogenase
$M_r$	Molecular weight
NADH	Reduced nicotinamide adenine dinucleotide
Nbs <sub>2</sub>	5,5'-dithiobis-(2-nitrobenzoic acid)
N.M.R.	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PDI	Protein disulphide isomerase
PEG	Polyethylene glycol
PPIase	Peptidyl prolyl cis-trans isomerase
$R_m$	Relative mobility
rpm	Revolutions per minute
Rubisco	Ribulose 1,5'- <i>bis</i> phosphate carboxylase
SDS	Sodium dodecyl sulphate
SRP	Signal recognition particle
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl) amino methane
u. v.	ultra violet



## Abstract

The unfolding and refolding of a number of oligomeric enzymes have been studied. These were: fumarase from pig heart, the NAD<sup>+</sup>-dependent isocitrate dehydrogenase from yeast, the citrate synthases from pig heart, *Acinetobacter anitratum* and *Thermoplasma acidophilum* and the chaperone protein GroEL from *Escherichia coli*.

In each case the unfolding by guanidinium chloride (GdnHCl) was monitored by enzyme activity (to detect possible perturbations at the active site), protein fluorescence (to detect changes in tertiary structure) and far u.v. circular dichroism (to detect changes in protein secondary structure). In general the losses in secondary and tertiary structure were found to run broadly in parallel, whereas the enzyme activity was lost at much lower concentrations of GdnHCl. This sensitivity to mild, denaturing conditions may reflect the greater flexibility of the active site compared with the molecule as a whole. Interestingly, the bacterial citrate synthases were activated in the presence of low concentrations of GdnHCl.

Following denaturation, refolding was initiated by lowering the concentration of GdnHCl by dilution or dialysis. Only the dimeric citrate synthases (from pig heart and *Thermoplasma acidophilum*) could be reactivated to a moderate extent using the dilution procedure; less than 5% reactivation was observed for the other enzymes. In the cases of fumarase, NAD<sup>+</sup>-dependent isocitrate dehydrogenase and the dimeric citrate synthases the degrees of reactivation following dialysis were significantly greater (approximately 50-75% of the native enzymes) than those obtained following the dilution procedure. Factors such as protein concentration and the inclusion of dithiothreitol in the dialysis or dilution buffer were found to influence significantly the extent of reactivation. The greater yield of reactivation of unfolded protein using the dialysis procedure probably reflects the ability of the enzyme to make the correct structural adjustments between intermediates when the concentration of GdnHCl is lowered gradually. In the case of *Thermoplasma acidophilum* the recovery of citrate synthase activity was much greater at 20 °C than at 55 °C (the optimal temperature for growth of this organism). This has implications for the folding process *in vivo* under the extreme growth conditions of thermophiles and possibly other extremophiles.

The hexameric citrate synthase from *Acinetobacter anitratum* and the tetradecameric chaperonin, GroEL could not be reactivated following denaturation. Far u.v. circular dichroism measurements on GroEL indicated that the native secondary structure of this protein was regained to a large extent.

*In vivo* a number of the proteins studied (fumarase and citrate synthase from pig heart and yeast NAD<sup>+</sup>-dependent isocitrate dehydrogenase) are translocated into mitochondria as precursors in a non-native state prior to processing, folding and assembly. The lack of complete refolding of the proteins studied in this work points to the existence of specialised mechanisms *in vivo* to promote efficient folding. Chaperone proteins have been implicated in the assistance of protein folding *in vivo*. Intriguingly, the studies on the inefficient refolding of the chaperonin GroEL support the proposal that this protein may fold *in vivo* by way of a “self chaperoning” mechanism.

*For my dear family and friends -with love*

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I thought of a few good quotes for this thesis "abandon hope all ye who enter"- Dante, was one of them but I thought it would discourage my examiners; "Of making many books there is no end-and much study is a weariness of the flesh" Ecclesiastes 12:12 was another but I suppose if there is no pain there is no gain -as they say.

## *Chapter one*

### **General Introduction**

## **1.1 Protein biosynthesis and the Central Dogma**

Proteins play crucial roles in most biological processes. All of the information required to generate the primary structure of a protein is encoded in the DNA or RNA of an organism. The function of the protein is dependent upon the conformation adopted by the protein following transcription and translation and is governed by its amino acid sequence. The sequence of events starting with the genetic material and leading to the biosynthesis of a functional protein is known as the Central Dogma. The amino acid sequence of a given protein can be determined from the sequence of bases of the genetic material from which it was encoded by application of the genetic code. One of the fundamental goals of the protein biochemist is to determine the way in which amino acid sequences specify the conformations of proteins.

## **1.2 The nature of the protein folding problem**

The method by which a protein attains a specific, folded, 3-dimensional structure remains a largely explored problem which has yet to be fully resolved. It has been known for some time that the conformation adopted by a particular protein is intrinsically governed by its amino acid sequence, however, the relationship between the primary sequence of a protein and its mechanism of folding requires further elucidation. Several workers have concentrated their efforts on obtaining a solution to the protein folding problem, the results of which have given rise to very interesting and informative speculations on the pathway of folding, not to mention an increasing body of evidence pointing to the contribution of other proteins which serve to assist in the folding process. The motives behind understanding protein folding are manifold. Not only would it solve an intellectual puzzle but it would also be of great use in the fields of biotechnology, molecular biology and medicine (Creighton, 1990).

The advent of recombinant DNA technology has re-emphasised the need to 'crack the protein folding code' (provided such a code really exists) mainly due to difficulties

encountered in obtaining correctly folded, functional products following the over-expression of recombinant proteins. The production of inactive, insoluble protein aggregates (such aggregates have also been termed “refractile bodies” and “inclusion bodies”) are perhaps not surprising considering that overexpression is often performed using a host organism which does not naturally synthesise the desired protein (Marston, 1986). In addition several other factors and/or proteins may be required for the successful folding of the protein which are absent from the milieu of the expression system. Such factors include pH, ionic concentration, post-translational modifying enzymes, enzymes involved in the catalysis and/or assistance of protein folding, for example, proteins with peptidyl prolyl cis-trans isomerase (PPIase) activity, protein disulphide isomerase (PDI) and a host of ‘helper’ proteins collectively known as chaperone proteins.

The influence of many of these factors on the successful folding of proteins *in vivo* and *in vitro* will be highlighted during the course of this chapter in an attempt to summarise the present school of thought on the mechanism of folding of oligomeric proteins.

### **1.3 Historical perspectives in protein folding**

In 1910 it was noted by Ramsden that ‘A dead frog placed in saturated urea solution becomes translucent and falls to pieces in a few hours’ (Pain, 1994). This finding, although utilising a somewhat mercenary approach, illustrates some of the early studies on the unfolding of proteins. Later work by Anson and Mirsky (1931) and Anson (1945) described the denaturation and subsequent refolding to yield functional protein of haemoglobin, trypsin, chymotrypsin and serum albumin (Dill, 1990; Pain, 1994). Anfinsen’s work on the successful, *in vitro*, refolding of ribonuclease A provided further support for the statement forwarded by Anson in 1945 that ‘proteins acquire their spatial structure autonomously in a spontaneous reaction’ (Jaenicke, 1993). In his conclusions, however, Anfinsen did not exclude the possibility of the involvement of other proteins in the folding process; in fact he suggested that “shuffling enzymes” could be involved in the catalysis of the rate-limiting steps in protein folding (Anfinsen, 1973). His insight has since been confirmed by the discovery of enzymes such as protein disulphide isomerases (Freedman, 1984) which catalyse the formation of correct



sets of disulphide bonds by promoting the reshuffling of incorrect disulphide pairs and peptidyl prolyl cis-trans isomerase (Fischer and Schmidt, 1990) which catalyses the isomerisation of Xaa-Pro peptide bonds and can accelerate the refolding of proline containing proteins *in vitro*.

#### **1.4 Methods of studying protein folding and refolding**

A number of techniques exist which facilitate the study of protein folding and serve to assist in the characterisation of the intermediate steps along the folding pathway/s. In refolding studies the protein is first denatured to effect unfolding and dissociation of domains and/or subunits. Denaturation can be achieved by several means including: extremes of pH and temperature; high pressure; chaotropic agents notably, GdnHCl and urea. The latter have been found (at high concentrations) to be more effective in disrupting the non-covalent interactions which are essential for maintaining the integrity of the native conformation of proteins. Furthermore, examination of proteins dissolved in GdnHCl suggests that its structure resembles that of a random coil at high concentrations of denaturant. By contrast, residual protein structure has been found to exist in some thermally or acid denatured proteins (Matthews, 1993). The refolding of proteins can be initiated by returning the protein to conditions from which the denaturing agent or factor have been removed or diluted. When the refolding of protein has been initiated its progress along the folding pathway can be monitored by techniques such as those described below in addition to those summarised in Table 1.1.

##### **1.4.1 Detection of folding intermediates**

Early folding events such as those which occur in the millisecond timescale following the removal of denaturant can be monitored by stopped-flow circular dichroism and/or stopped-flow fluorescence studies. These techniques are now widely used to monitor the so called burst phase of protein folding incorporating changes which occur in the very early stages of folding. For example Kuwajima and co-workers (1987) have been able to show using stopped flow c.d. that a substantial fraction of secondary structure is formed in  $\beta$ -lactoglobulin within the millisecond time scale.

NMR spectroscopy studies have also been used to monitor early events in the folding

**Table 1.1 Techniques which can be used to study the refolding of proteins**

<b>Property studied</b>	<b>Technique</b>	<b>Reference</b>
Formation of native state (biological activity)	Kinetics of reactivation	Chan <i>et al.</i> (1973)
	Kinetics of ligand binding	Garel & Baldwin (1973)
	Inhibitor binding	Kato <i>et al.</i> (1982)
<b>Protein conformation</b>		
Environment of tryptophan and tyrosine residues	Fluorescence	Tiepel & Koshland (1971)
Formation of secondary and tertiary structure	Far u.v. c.d.	Labhardt (1986)
	Near u.v. c.d.	
Environment of aromatic residues	Absorbance	Ikeguchi <i>et al.</i> (1986)
Formation of persistent hydrogen bonds, burial from solvent at individual residues	Hydrogen exchange labelling and NMR	Kim (1986)
		Roder <i>et al.</i> (1988)
	Susceptibility to proteolysis	Radford <i>et al.</i> (1992)
Exposure of hydrophobic surfaces	ANS binding	Girg <i>et al.</i> (1981)
		Ptitsyn <i>et al.</i> (1990)
State of association	Cross-linking studies	Hermann <i>et al.</i> (1981)

process. An example of the usefulness of this technique can be provided by examination of the results obtained by Radford *et al.* (1992) and Dobson *et al.* (1994) in their studies of the folding of hen lysozyme. In this work the technique of hydrogen exchange labelling was employed combined with two-dimensional NMR. The basis of the hydrogen exchange experiment is the measurement of the rate of exchange between the solvent and amide (or side chain) hydrogens. By using deuterium the exchange can be readily quantified. Hydrogens which are protected from exchange can be assumed to be involved in intramolecular hydrogen bonds or buried from the solvent in the protein core. Both of these explanations imply the formation of secondary and possibly tertiary structure. Furthermore this technique allows the stability of individual residues to be monitored during the folding process and gives a measure of the stability of folding intermediates in terms of the native conformation of the protein.

The aforementioned probes of folding intermediates combined with those listed in Table 1.1 are most useful when used together to build a picture of stages throughout the folding process. For example in the experiments of Radford *et al.* several of the above methods were employed to yield interesting results on the folding of hen lysozyme (Radford *et al.*, 1992); it should be noted however, that the four disulphide bonds were maintained during the unfolding/folding procedure. The NMR studies revealed that about half of the amides, corresponding to those of the  $\alpha$ -domain which contains four  $\alpha$ -helices, are protected from hydrogen exchange within 200 milliseconds while the remaining amides, most of which correspond to those found in the  $\beta$ -domain which contains the  $\beta$ -sheet, are not protected until at least 1 second. Stopped-flow c.d. studies were able to show that a large proportion of secondary structure forms within a few milliseconds of the refolding process. It was concluded that the rate of secondary structure formation is more rapid than the rate at which protection from hydrogen exchange occurs, suggesting that protection is afforded by the stabilisation of the pre-existing helical structure. Examination of the stopped flow kinetics of events in the near u.v. region of the spectrum (data were recorded at 289nm corresponding to the c.d. of tryptophan residues) indicated the absence of tertiary structure after 200ms despite the preponderance of secondary structure. It was suggested that the folding intermediate is in a state reminiscent of the molten globule at this stage of the folding process. Studies using the fluorescent dye 1-anilino naphthalene sulphonate (ANS) which binds to the

hydrophobic regions of proteins, showed that the enhancement in fluorescence in the early stages of protein folding was  $\leq 7$ -fold greater than that of native protein. This finding suggests that the protein is in a loosely packed state and supports the existence of a molten globule at this stage in the proceedings (Dobson *et al.*, 1994). Further work involving the intrinsic fluorescence of refolding lysozyme and the binding of a fluorescent inhibitor were employed by these workers to monitor the environment of the tryptophan residues and the formation of the active site respectively.

Experiments involving the genetic manipulation of proteins have been paramount in elucidating the contribution of individual amino acid side chains to the structure of the folding protein. Site directed mutagenesis is now a widely used approach which allows the selective replacement of one particular amino acid with another, the effect of which can be used to discern the properties of the replaced amino acid in terms of folding and structure (Fersht and Winter, 1992). Further genetic engineering techniques include: the deletion of certain regions of the polypeptide chain to assess the importance of that region to the folding process (Shortle and Meeker, 1989) and the expression of isolated regions of the polypeptide chain, for example individual domains, to examine the ability of the domain to fold as an independent folding unit. Such studies have been employed by Missiakas *et al.* (1990) and Herold *et al.* (1991) in the construction of the two domains of phosphoglycerate kinase and the coenzyme binding domain of aspartate aminotransferase respectively. In each case the protein fragments obtained were capable of autonomous folding although the stability of the isolated domains was lower than those of the corresponding domains present in the intact protein.

## **1.5 The rationale behind the study of protein folding *in vitro***

Proteins are synthesised *in vivo* in a stepwise manner which involves the sequential condensation of individual amino acids to produce a growing polypeptide chain. The process occurs on the ribosome and is unidirectional in that synthesis occurs from the N-terminus to the C-terminus of the nascent chain at a rate of 1-10 residues per second. The folding of proteins has been shown to occur both during protein synthesis (co-translational folding) and following the termination and release of the polypeptide chain

from the ribosome complex (post-translational folding). Although evidence has been obtained *in vivo* demonstrating both co- and post-translational folding, the speed of folding has been found to be extremely fast such that its examination is rendered virtually impossible. Given the difficulty in monitoring the folding process *in vivo* many workers have turned to the study of protein unfolding and refolding *in vitro* to gain insight into the mechanism of protein folding *in vivo*. Whether or not the results of such studies are applicable to the situation *in vivo* is most certainly dependent on the protein studied and the conditions employed. However, certain criteria exist which suggest that the *in vitro* process can be related to the situation in the cell. Primarily, it has been found that the rate of successful refolding of a denatured protein *in vitro* (in the seconds to minutes range) is comparable with the time taken for a protein to fold *in vivo* [calculated by Tsou (1988) to be of the order of about 30 seconds]. For example, *Staphylococcus* nuclease and aldolase have been shown to refold, *in vitro*, within 1 second (Epstein *et al.*, 1971) while myoglobin takes up to 10 seconds to refold following acid induced unfolding (Shen and Hermans, 1972). Secondly, with some exceptions examination of the protein products following folding in the cell and refolding *in vitro* indicate that they are virtually indistinguishable (in spite of differences in their folding milieu). The exceptions to this observation tend to be larger oligomeric proteins and proteins which are normally targeted to cellular organelles prior to folding. Given the complexity of many larger proteins and competing side reactions, notably aggregation, it is not surprising that the refolding of such proteins *in vitro* is unsuccessful.

There remain many differences between the refolding of proteins in the test tube and the folding of nascent polypeptides in the cell. As mentioned previously the refolding of unfolded proteins starts with the complete polypeptide chain while the folding of nascent polypeptide can occur during translation. Furthermore, the cellular concentration in which the nascent polypeptide folds is extremely high compared with the limited range of protein concentrations at which refolding can occur successfully *in vitro*.

## **1.6 General properties of protein folding**

The *in vitro* refolding of several proteins have been defined from which models of

folding have been proposed notably; bovine pancreatic trypsin inhibitor protein, BPTI (Creighton, 1990); barnase (Ferscht *et al.*, 1992); lysozyme (Dobson *et al.*, 1994). Some of these pathways will be discussed in this section in addition to the concept of a general model for protein folding.

### 1.6.1 Hierarchy of protein structure

Before examining some pathways of protein folding it is worthwhile considering the hierarchical classification of protein structure. Linderstrøm-Lang was instrumental in defining the idea of “hierarchical condensation” in 1952 which later formed the basis of the concept of the pathway of protein folding and the attainment of the stable, functional protein (Jaenicke, 1993).

Protein structure can be defined in terms of primary, secondary, tertiary and quaternary structure. The primary structure of a protein refers to the amino acid sequence and any posttranslational modification of the polypeptide chain encoded by the genetic material of the genome. In some proteins the amino acid sequence of the nascent polypeptide may differ from that of the mature protein. This situation arises, for example, in the case of proteins which contain N-terminal presequences and are targeted to other cellular organelles prior to folding. On arrival at their correct destination the presequences are cleaved (Pfanner and Neupert, 1990). The secondary structure is the local, regular structure adopted by the polypeptide backbone. Such structures include the formation of  $\alpha$ -helix,  $\beta$ -sheet and random coil, the formations of which are influenced by the amino acid sequence and solvent conditions. Clusters of secondary structure are referred to as supersecondary structure which have been described as intermediates between secondary and tertiary structure (Stryer, 1988). The folding of the polypeptide to yield 3-dimensional structure is also termed the tertiary structure of a protein and includes the formation of compact regions or units called domains. Proteins which contain more than one polypeptide chain undergo further structural organisation resulting in quaternary structure. Each polypeptide in such proteins forms a subunit and the spatial arrangement and association of these subunits characterises the quaternary structure of a given protein.

### **1.6.2 Intrinsic factors involved in protein folding**

The amino acid sequence of a protein determines its native conformation. Given the theoretical number of possible conformations which a typical protein can adopt it is of great interest to establish the factors which favour one particular conformation over the others bearing in mind the impossibility (within a finite time scale) of attaining the correct 3-dimensional structure by a random search. On the basis of such calculations, Levinthal (1968) postulated that there must be protein folding pathways to accelerate the process.

The question of which properties of the amino acid sequence contribute to the driving force required to guide the nascent or refolding polypeptide chain to its native conformation has been examined in some detail. Contributory forces to this process include: next neighbour and short range interactions (such as hydrogen bonding, hydrophobic and Coulomb interactions, van der Waals forces); optimum packing density (attainment of minimum hydrophobic accessible surface area); entropic effects pertaining to the release of water during the process of 'hydrophobic collapse' and subunit assembly (Dill, 1990; Jaenicke, 1993). The entropy gain due to this 'hydrophobic collapse' is perhaps the most important non-covalent interactions involved in stabilising a protein in its native conformational state in addition to intramolecular hydrogen bonding and van der Waals interactions. The latter occur between non-polar groups of atoms which have been shown, using model systems to have a tendency to remain in non-polar environments. Van der Waals bonds are less favourable in an aqueous environment. Ion pairing is common on the surfaces of proteins as shown by the X-ray crystal structures of known proteins. It has been demonstrated that although interactions between ions are not considered a dominant contributor to protein stability variations in amino acid sequence which affect ion pairing (Coulomb interactions) can alter the stability of a protein ( Dill, 1990).

### **1.6.3 Extrinsic factors involved in protein folding**

In recent years it has come to light that other auxiliary factors are involved in the folding of proteins in the cell. These findings do not detract from the fact that folding is directed by the amino acid sequence of a polypeptide-they suggest that the folding process is aided by other cellular factors. These factors can be divided into two categories:

(i) those which catalyse protein folding and, (ii) those which facilitate protein folding by acting as a 'chaperone' at some stage in the process (see section 1.7).

#### 1.6.3.1 Catalysis of protein folding by PDI

Protein disulphide isomerase (PDI) is a highly abundant eukaryotic protein which is found mainly in the lumen of the endoplasmic reticulum of cells which synthesise disulphide containing proteins (such proteins are generally those destined for secretion or cell surface proteins). Indeed, it has been shown that the enzyme amounts to about 2% of the entire protein content of rat liver microsomes (Freedman, 1984, 1989). The carboxy-terminal sequence of mammalian PDI is KDEL which is characteristic for proteins resident in the ER (Munro and Pelham, 1987). The corresponding sequence for PDI found in the ER of yeast has been shown to be HDEL. The mammalian enzyme is a homodimer comprising identical subunits of  $M_r$  57 000. The active site domains,  $\alpha$  and  $\alpha'$ , of PDI are similar in sequence to each other (47%) and have been shown to be homologous to thioredoxin, a small (12 kDa) dithiol-disulphide oxidoreductase which is involved in a number of oxidoreduction processes (Freedman *et al.*, 1994).

PDI has been shown to catalyse thiol/disulphide exchange encompassing net oxidation, reduction and rearrangements. It catalyses the *in vitro* reoxidation of a wide variety of proteins under suitable solvent and redox conditions. For example it has been shown that for the folding of RNase A, in the presence of glutathione, the catalysis of the formation of disulphide bonds by PDI requires a redox buffer which contains both reduced and oxidised components (Lyles and Gilbert, 1991). Weissman and Kim (1993) have recently reported that PDI is capable of increasing the rates of folding of kinetically trapped BPTI folding intermediates by a factor of 3000-6000 - illustrating the efficiency of the enzyme in catalysing disulphide bond rearrangements.

The confirmation of the important role of PDI *in vivo* has been provided by a number of studies. For example Roth and Pierce (1988) using cross-linking studies as a probe for proteins found to be associated with PDI in lymphocytes, reported the association of nascent immunoglobulin chains with the enzyme. Further evidence from *in vivo* studies were provided by Bulleid and Freedman (1988) who demonstrated, using dog pancreas microsomes depleted of soluble proteins, that although their *in vitro* translation system



was capable of synthesising and translocating the wheat storage protein,  $\gamma$ -gliadin, the disulphide bonds of the protein were incomplete. It was found that the addition of purified PDI to the microsome system allowed the formation of disulphide to proceed correctly. Yeast PDI has been found to be essential for the organisms viability; the disruption of the gene encoding this enzyme precludes spore germination (Gething and Sambrook, 1992; Freedman *et al.*, 1994).

PDI has been found to be present as a component of at least two other enzyme systems. The enzyme prolyl-4-hydroxylase which is involved in the modification of proline residues in newly synthesised collagen molecules has been shown to be a tetramer comprising two types of subunit,  $\alpha_2\beta_2$ . It has been demonstrated that while the  $\alpha$ -subunits form the active site of the enzyme the  $\beta$ -subunits are identical to PDI (Freedman *et al.*, 1994). As yet the role of PDI in this system has not been clarified although preliminary studies using enzyme depleted of the  $\beta$ -subunits have indicated that it may be involved in maintaining the solubility and integrity of the tetramer (Vuori *et al.*, 1992). PDI has similarly been found to be identical to the  $\beta$ -subunit of a triacylglycerol transfer protein (MTP) which is involved in the incorporation of lipids into nascent core lipoproteins in the ER. It has been suggested that the role of PDI in this complex is similar to that postulated for that of the prolyl-4-hydroxylase complex.

#### **1.6.3.2 Catalysis of protein folding by PPI**

In proteins there are two possible isomeric forms of the amide bond, cis and trans, which differ by a  $180^\circ$  rotation about the carbonyl carbon to amide nitrogen C-N bond. X-ray crystallographic studies have shown that for the majority of amide bonds the trans conformation is favoured. The imide bond characteristic of the linkage between proline residues and the preceding amino acid has been shown to contain both cis and trans conformations. It has been demonstrated that many proline-containing proteins contain both cis and trans imide linkages, although the latter are more prevalent. RNase A, ribonuclease T1 and thioredoxin are examples of proteins which contain one or more cis-prolines (Nall, 1994). The refolding of certain proteins requiring Xaa-Pro isomerisations *in vitro* has been shown to be dominated by slow steps on the pathway probably due to the high activation energy of cis-trans isomerisations (Lang *et al.*, 1987; Fischer *et al.*, 1989; Stewart *et al.*, 1990).

In 1984 peptidyl prolyl cis-trans isomerase (PPIase) was discovered by Fischer and his co-workers. This enzyme was shown to catalyse the 180° rotation about the C-N linkage of the peptide preceding proline *in vitro*. Since nascent polypeptides are presumably translated in the all trans conformation despite the existence of cis prolyl bonds in native proteins it has been postulated that PPIase, a highly ubiquitous enzyme, acts *in vivo* as a folding catalyst involved in the cis-trans or trans-cis isomerisation steps necessary for the production of functional proteins. Fischer *et al.* (1989) were involved in the characterisation of PPI and these workers demonstrated that the enzyme from porcine kidney is identical to bovine cyclophilin which is inhibited by the immunosuppressive agent cyclosporin A. Indirect evidence for a role for PPI in the cellular folding process is provided by the demonstration that the rate of collagen triple helix formation, which is limited by proline cis-trans isomerisation, is delayed following treatment of chicken and human fibroblasts with cyclosporin A - an inhibitor of PPI (Steinman *et al.*, 1991).

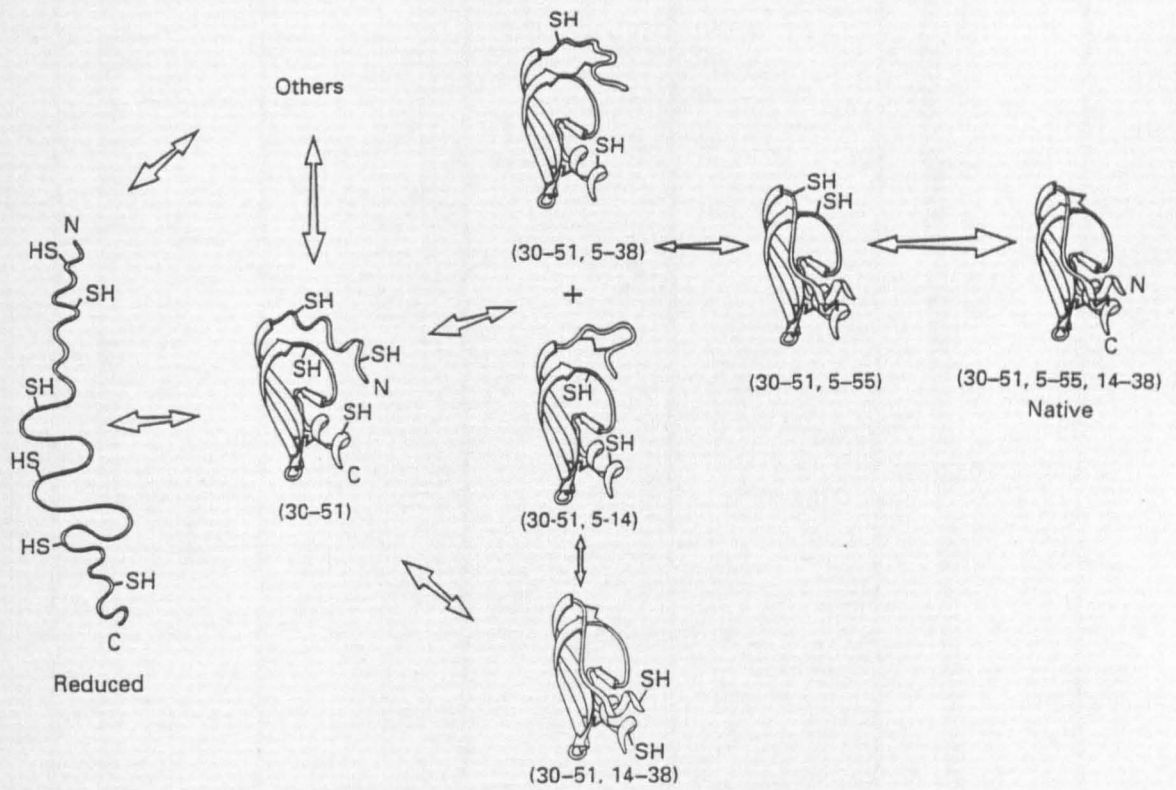
#### **1.6.4 Folding of monomeric proteins**

The folding of proteins consisting of a single polypeptide chain has been examined extensively by several workers in an attempt to gain insight into the pathway of folding. Such proteins exist as either single domain proteins or multidomain proteins. Bovine pancreatic trypsin inhibitor (BPTI) is an example of a monomeric protein with a single domain. The enzyme is stabilised by three disulphide bonds (between cysteine residues 5-55, 14-38 and 30-51) the reduction of which leads to the reversible unfolding of the protein. The refolding of this small protein has been studied in detail by Creighton *et al.* (1990) and Weissman and Kim (1991). These workers were able to examine the nature of the folding intermediates by trapping the disulphide bonds of the refolding protein at a given time in the process. Iodoacetate was used in the trapping procedure by virtue of its reactivity with thiol groups; irreversibly blocking the formation, interchange or breakage of disulphide bonds in the protein (Creighton *et al.*, 1990). The species trapped in this manner were analysed by non-denaturing polyacrylamide gel electrophoresis and ion-exchange chromatography (Weissman and Kim used acid quenching to trap the disulphides and reversed phase high performance liquid chromatography for the analysis of trapped species). Approximately 60% of the species analysed contained the disulphide bond between cysteine 30 and cysteine 51; remaining

disulphide species contained disulphides at residues 5-30, 5-51, 30-55 and 5-55. These studies revealed the existence of the 30-51 disulphide in the isolation of all subsequent intermediates indicative of its stability and suggesting an early role in the folding process. It was found that the successful folding pathway proceeds from this intermediate, as illustrated in Figure 1.1. Indeed, structural analysis indicated that the major  $\alpha$ -helix and  $\beta$ -sheet components of the native conformation appear to exist at this early stage of the folding pathway. The intramolecular rearrangement of the intermediate two disulphide species, 30-51, 5-14 and 30-51, 5-38 to yield the species, 30-51, 5-55 was found to be the rate-limiting step in the refolding process although it is the most energetically favourable pathway towards the final native conformation of BPTI. The formation of this native-like, disulphide species also brings the remaining two cysteine residues into proximity allowing the rapid formation of the final disulphide bond.

The refolding of multidomain monomeric proteins has also been examined extensively in recent years and it has been shown that the complexity of the folding mechanisms is intermediate between that of single domain, monomeric proteins and multi-subunit proteins. For example penicillinase A consists of three domains (separable by proteolysis) which have been shown to act as independent folding units. Refolding experiments, following denaturation, indicate that the regain of secondary structure is a rapid event while the regain of tertiary structure is a relatively slow process. When the protein is cleaved using cyanogen bromide three fragments are produced which can associate (also referred to as "domain pairing") with each other to form native-like structure (recognisable by antibodies towards native protein) although the enzyme remains inactive. The importance of domain pairing was highlighted when it was shown that the penicillinase A complex, formed following cleavage, is capable of undergoing reversible unfolding in GdnHCl; thus illustrating the influence of domain association on the stability of the folded structure (Adams *et al.*, 1980).

There is evidence to suggest that the rate limiting step in the folding of large single chain proteins is the pairing of already folded domains. For example, in the case of octopine dehydrogenase it has been shown that the refolding of the enzyme in terms of secondary and tertiary structure yields an intermediate product which, although inactive, is virtually indistinguishable from the native protein as judged by circular dichroism and



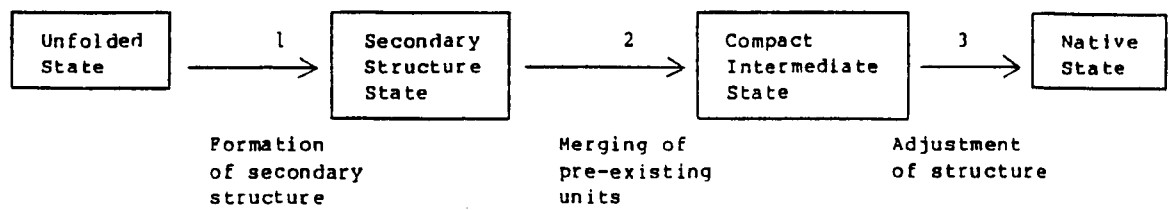
**Figure 1.1 Refolding pathway of bovine pancreatic trypsin inhibitor (from Creighton, 1990, 1994)**

fluorescence studies. The rate of reactivation was found to decrease when the viscosity of the reactivation solvent was increased. It was concluded from these observations that domain pairing rather than domain folding contributes to the slow rate determining step which precedes reactivation of the enzyme (Zettlmeissl *et al.*, 1984; Jaenicke, 1987). The negative effect of viscosity on the pairing of domains can be explained by the restriction imposed by such a medium on the movement of the domains relative to one another.

Experimental observations indicate that during the reactivation of certain proteins an intermediate can accumulate, consisting of at least some folded domains which have not yet interacted (or 'paired') prior to the slow domain pairing step (Garel, 1994). These Folded, Unpaired Domains which have been described as the 'FUD' intermediate can pose problems during the reactivation step due to the generation of kinetic competition between folding and aggregation. Prior to domain pairing the specific association site/s on an individual domain, are generally free and can be involved in either intramolecular binding to the correct domain partner or intermolecular binding with the domain of another chain. The latter can, in turn, lead to the formation of a large intermolecular species which precipitates in the form of an aggregate (Garel, 1994). This non-productive side reaction complicates the protein refolding process and necessitates the manipulation of experimental conditions such that aggregation is not favoured. This can often be achieved using low protein concentrations in the renaturation studies (Jaenicke, 1987).

#### **1.6.4.1 General model of protein folding**

These findings in addition to those outlined in section 1.4.1, describing some of the folding events of lysozyme (which contains two domains) and a large number of other studies (reviewed in Creighton, 1992) are examples of monomeric proteins which have been shown to follow a pathway of folding. A general model for the folding of such proteins has been proposed (Ptitsyn, 1973; Kim and Baldwin, 1982) which has been named the "Framework model". This model suggests that folding proceeds in a stepwise process as outlined in Figure 1.2.. It has been suggested that folding proceeds in three main stages (Ptitsyn, 1991): (i) formation of 'fluctuating embryos' of regions with secondary structure in an otherwise unfolded chain; (ii) hydrophobic collapse of



**Figure 1.2 A general model for the folding of monomeric proteins**

Stages 1, 2 and 3 involve predominantly hydrogen bonds, hydrophobic interactions and van der Waals interactions respectively.

these regions into an 'intermediate compact structure'-also described as a 'molten globule' (Ohgushi and Wada, 1983) and, (iii) adjustment of this intermediate structure to the unique native structure.

The concept of an 'intermediate compact structure' more popularly termed the molten globule has been the focus of attention in the study of protein folding for a number of years (reviewed by Kim and Baldwin, 1990; Ptitsyn, 1992; Christensen and Pain, 1994). A number of criteria exist which characterise the molten globule state: (i) the intermediate is condensed with a Stokes radius equal to or slightly (about 10%) greater than that of the native protein; (ii) it exhibits substantial secondary structure as exemplified by far-u.v. circular dichroism studies; (iii) there is a lack of persistent tertiary structure compared with the native protein; (iv) the molecules are prone to aggregation due to the exposure of non polar groups and, (v) in the case of enzymes the intermediate is inactive.

#### **1.6.5 Refolding of multi-subunit proteins**

The study of the folding of multi subunit proteins is intrinsically more complex than that of monomeric proteins; however, the principles of association and pairing of domains in monomeric, multidomain proteins are similar to those displayed by the subunits of larger proteins. Proteins vary considerably in terms of size and molecular weight. It is generally the case that the larger the protein the increasing likelihood that it will exist as a multi-subunit protein. The advantages of the emergence of multi-subunit structures are manifold and provide enhanced opportunities for the regulation, productivity and stability of functional proteins. For example the association of the four subunits of aspartokinase-homoserine dehydrogenase (AK-HDH) gives rise to an enzyme which is capable of catalysing two steps in the biosynthesis of threonine (Dautry-Varsat and Garel, 1981); although the aspartokinase and homoserine-dehydrogenase activities can be physically separated the enzyme is only subject to regulation by threonine when it is associated in the native tetrameric state.

Several techniques have been employed to assess the dissociation and association of subunits in oligomeric proteins. Since the quaternary structures of such proteins are often more labile than their tertiary structures it is possible to dissociate subunits under

certain denaturing conditions without affecting their folded conformation. The rate of reactivation of such species, following removal of the dissociating agent, can be compared with the rate of refolding and association of the completely unfolded protein. This technique has been used to show that the association of two dimers into a tetramer is the rate limiting step in the folding of proteins such as lactate dehydrogenase (Jaenicke, 1987) and phosphofructokinase (Deville-Bonne *et al.*, 1989). The presence of different subunit species at a given time in the refolding/association process can be examined using the techniques of chemical cross-linking combined with sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE). The cross-linking reagent glutaraldehyde has been used effectively for such purposes.

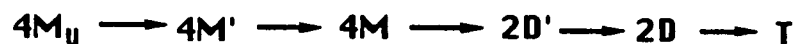
#### 1.6.5.1 Examples of the association of some oligomeric proteins

Bearing in mind the complexity and variety of quaternary structures adopted by different oligomeric proteins it is not surprising that a general scheme for folding and assembly has not been defined. However, a number of studies on the unfolding and refolding of oligomeric proteins indicate that the rate-limiting step of the process involves the association of subunits to yield the functional protein.

Aspartokinase homoserine dehydrogenase is a tetrameric enzyme with bi-functional enzymatic properties as mentioned previously (section 1.6.5). Fragmentation of the enzyme by limited proteolysis and genetic manipulation techniques have allowed the unfolding and refolding of the individual subunits to be examined both separately and together with the other subunits of the protein (Dautry-Varsat and Garel, 1981). These studies have shown that an active monomer is responsible for the aspartokinase activity the formation of which involves at least two steps. The first step is the rapid folding of the monomer ( $M_u$ ) to yield native-like secondary and tertiary structure ( $M'$ ) as judged by circular dichroism and fluorescence studies; the second step is the pairing of the domains of the monomer to yield active monomer ( $M$ ) with aspartokinase activity. As mentioned in section 1.6.4, this constitutes a crucial step in folding pathways since incorrect folding can give rise to the formation of aggregates. The correct formation of the dimers ( $2D'$ ) can be detected by the appearance of homoserine dehydrogenase activity (the inhibition of the dehydrogenase activity by threonine is a measure of the correct 'pairing' of the two dimers - $2D'$ ). The final step in the folding pathway of this



protein results in native enzyme (T) with aspartokinase and homoserine dehydrogenase activities which can both be inhibited by the presence of threonine. A scheme for this folding pathway is illustrated below:



The refolding of tetrameric lactate dehydrogenase from pig heart and skeletal muscle has been examined in some detail by Jaenicke and co-workers (Jaenicke, 1987). Following unfolding, in GdnHCl, these workers used the technique of chemical cross-linking to investigate the subunit species present at various stages in the folding process. It was found that dimeric intermediates accumulate during the folding of lactate dehydrogenase indicating that the formation of the tetramer is rate limiting in this pathway. Kinetic analyses of the steps along the pathway indicate that the equilibrium between the monomer and dimer is rapid [ $K = 3 \times 10^8 \text{ (mol dm}^{-3}\text{)}$ ] while the association of dimers to yield the active tetramer is considerably slower [ $k_2 = 3.0 \times 10^4 \text{ (mol dm}^{-3}\text{)}^{-1} \text{ s}^{-1}$ ]. The kinetic scheme for this reaction is given below:



where  $M''$  represents unfolded monomer,  $M'$  and  $M$  represent different conformational states of the monomer,  $D$  corresponds to the dimer and  $T$  is the tetramer.

Circular dichroism measurements revealed that within the manual mixing time of the experiment (approximately 10 seconds) at least 75% of the native secondary structure is regained, corresponding to the monomeric species,  $M'$ , in the above refolding scheme. The slow transition between  $M'$  and  $M$  (rate constant =  $0.8 \times 10^3 \text{ s}^{-1}$ ) is presumably due to the pairing of domains within the monomer. Further characterisation of the dimer was achieved by trapping the dimeric intermediate by addition of thermolysin one minute

after the initiation of refolding. This treatment was shown to cause the removal of about 10 amino acids from the N-terminal arm of each subunit. X-ray crystallography studies have shown that this 'arm' is involved in the interactions between the dimers in the native enzyme. Girg *et al.* (1983) showed that both the proteolytic dimers and the dimer intermediates of the reassociation pathway exhibited about 50% of the native fluorescence and circular dichroism similar to that of the functional enzyme. The conformation adopted by both sets of dimers was capable of binding the triazine dye, procion green which binds at nucleotide binding sites- implying the existence of this site at the dimer level in the folding pathway. Furthermore it was shown that in the presence of  $(\text{NH}_4)_2\text{SO}_4$  ions the dimers possess about 40% of the native catalytic activity presumably due to a "tightening" of the rather flexible globular structure of the dimeric intermediates.

#### **1.6.5.2 The specificity of association**

An important consideration of the folding process is the degree of specificity in the association of subunits given the large number of folding proteins present in the cell. Association steps involve interactions between subunits at specific recognition sites. Numerous studies using a variety of different and similar proteins have shown that there is a high degree of specificity in the association of subunits to yield native protein. Cook and Koshland (1969) demonstrated the successful reactivation, following unfolding, of a number of enzymes in the presence of mixtures of refolding multi-subunit proteins. London *et al.* (1974) showed that the refolding of tryptophanase was similar in the presence and absence of other proteins such as bovine serum albumin or the proteins of a cellular crude extract. Examination of the refolding of a mixture of the isoenzymes of lactate dehydrogenase, however, does reveal the presence of hybrid species reflecting the conservation of the recognition sites and three-dimensional structure of these enzymes.

#### **1.6.5.3 Difficulties in refolding oligomeric proteins *in vitro***

Several studies have shown that the refolding of a number of proteins is less than 100% efficient. Proteins such as glutamate dehydrogenase (West and Price, 1988) and ribulose biphosphate carboxylase/oxygenase have yet to be successfully refolded *in vitro* following complete dissociation and unfolding. Other proteins, including translocated

proteins (section 1.7) and large proteins which contain more than 300 amino acids in their polypeptide chain have proven difficult to refold *in vitro*. A number of factors may contribute to the difficulties encountered during the refolding of these proteins. The tendency of proteins which contain long polypeptide chains to form domains, the pairing of which often constitutes a rate determining step, gives rise to the possibility of the incorrect intermolecular domain pairing with the domains of other chains leading to the formation of aggregates (section 1.6.4). The association of the subunits of oligomeric proteins can also be affected by competing side reactions. At high protein concentrations the formation of aggregates is favoured since the pathway leading to aggregation is a multimolecular reaction, the rate of which increases as a function of increasing protein concentration. At low protein concentrations refolding is more efficient, however other factors may interfere with the folding process, such as adsorption, degradation or chemical modification of amino acid side chains (Price, 1994). In general it is necessary to establish the optimal *in vitro* refolding conditions of a given protein in terms of concentration in order to minimise the aforementioned side reactions.

### **1.7 The targetting and folding of translocated proteins *in vivo***

In eukaryotes the majority of polypeptides are synthesised in the cytoplasm before being translocated to a particular destination in the cell or to an extracellular location. The process by which a protein is directed to a particular location has been the focus of a great deal of attention in the last decade. Over this time it has been revealed that detailed mechanisms and other factors are involved in the various processes which result in protein targeting to the correct destination.

The majority of proteins which are translocated across biological membranes (such as the membranes of the endoplasmic reticulum, mitochondria, chloroplasts, nucleus and the plasma membrane) are synthesised in the form of precursor proteins which have cleavable amino-terminal signal sequences. These sequences are important in specifying the destination of certain proteins.

Proteins which are processed in the endoplasmic reticulum prior to secretion or delivery

to a particular location have been shown to possess an N-terminal signal peptide which, following synthesis, is recognised by the signal recognition particle (SRP). SRP binds to the signal peptide thus forming a complex with the ribosome, messenger RNA and the nascent peptide. During this process peptide elongation is temporarily arrested. The SRP complex becomes associated with the membrane of the ER by interaction with a docking protein which is also known as the SRP receptor. Displacement of the ribosome machinery from the SRP molecule to the receptors for ribosomes and nascent signal peptides (Rapoport, 1990) allows the SRP to be released from the docking protein and peptide synthesis to resume. The nascent polypeptide chain has been shown to be co-translationally 'threaded' into the lumen of the ER where it can be processed further. The signal sequence is generally cleaved, following entry into the lumen, by a large membrane bound signal peptidase. Studies on the import of proteins into the lumen of the ER have revealed the importance of a heat shock protein, hsp70, in the translocation and protein sorting and folding processes (Deshaies *et al.*, 1988; for review see Gething and Sambrook, 1992).

A second mechanism of transport of proteins into the ER has been proposed which does not involve the action of ribonucleoparticles and their receptors. This ribonucleoprotein-independent pathway was demonstrated by Schlenstedt *et al.* (1990) who observed that small precursor proteins such as procecropin A (ppcecA) are efficiently transported into salt-washed or trypsinized microsomes which are consequently devoid of SRP and docking protein. In this transport mechanism proteins are inserted into the lumen posttranslationally; one ATP-dependent reaction is involved in maintaining the polypeptide in a transport competent state and another facilitates insertion of the protein into the membrane. The signal peptide of the precursor proteins together with a group of proteins collectively known as chaperone proteins are responsible for maintaining the transport competent state of the protein prior to transport into the ER; the chaperone proteins have been shown to be capable of ATPase activity. It has been suggested that the transport-competent state is probably a molten globular state (Wiech *et al.*, 1990; Dierks *et al.*, 1993).

The majority of proteins which are contained in the mitochondrion are encoded by nuclear genes, synthesised in the cytoplasm and subsequently translocated into the

particular mitochondrial sub compartments (Horwich, 1990; Pfanner and Neupert, 1990). The process by which proteins are imported into mitochondria is similar to that which directs proteins into the ER. Proteins destined for the various mitochondrial subcompartments contain N-terminal signal sequences which are usually cleaved by proteolysis following translocation across the membrane. These presequences vary in length from 12 to 70 residues and have been shown to be rich in basic residues, hydroxyl -containing serine and threonine residues and small hydrophobic residues (Schatz, 1987). The presequences have been shown to bind to receptors on the surface of the mitochondrial outer-membrane and to trigger the movement of the amino-terminus across the inner membrane. This insertion process is aided by the presence of proteinaceous (Neupert *et al.*, 1990) translocation “pores” which are present in both the outer and inner membranes at “translocation contact sites” - regions where the outer and inner membranes are in close contact (Schleyer and Neupert, 1985; Hwang *et al.*, 1991; Rassow and Pfanner, 1991). Martin *et al.* (1991) suggested that the electrical membrane potential across the inner membrane (which has been shown to be necessary for translocation across the inner membrane to occur) attracts the positively charged targeting, presequence of the non-native protein resulting in the electrophoretic transfer of this part of the amino-terminus through the inner membrane. When the protein has reached the matrix the targeting sequence is cleaved and the protein can undergo folding to yield native protein or be sorted to other mitochondrial compartments.

In recent years it has been demonstrated that the energetics of translocation in addition to the mechanisms involved in maintaining the protein in a ‘translocation-competent state’ prior to and during translocation as well as the folding of the protein following importation are closely linked to a large group of proteins known as heat shock proteins or molecular chaperones (Ellis, 1987; Hemmingsen *et al.*, 1988; Cheng *et al.*, 1989; Manning-Kreig *et al.*, 1991; Neupert and Pfanner, 1993). A great deal of experimental evidence supports a role for both heat shock proteins (hsp) 70 and 60 in these translocation and/or unfolding/folding processes. Studies on the yeast, *Saccharomyces cerevisiae* have identified eight types of hsp 70s of which one is localised in the mitochondria ; the Ssc1p or mitochondrial (mt-) hsp 70 (Craig *et al.*, 1989). Deletion of the gene encoding mt-hsp 70 (SSC1) results in cell death. Kang *et al.* (1990) demonstrated using a temperature sensitive mutant of the SSC1 gene that mitochondrial

precursor polypeptides accumulated in the cytosol at non-permissive temperatures. Following a short time interval these precursor proteins were found to be associated with the mitochondria but they were sensitive to proteases indicating that translocation into the mitochondria does not occur under such conditions. Further analysis implicated the involvement of mt-hsp 70 in both the translocation of precursors across the mitochondrial membranes into the matrix (after the signal sequence has “triggered” the translocation process by moving across the inner membrane, as described previously) and in the folding of precursors in the mitochondria. The requirement for ATP in both the matrix and the cytosol has been shown (Hwang and Schatz, 1989) which is consistent with the need of the mt-hsp70 for ATP in the binding and release mechanism involved in the interaction with polypeptide chains as they are translocated into the matrix and processed further.

Another heat shock protein, notably hsp60 (or chaperonin 60-cpn 60) has been found to be involved in the folding of polypeptide chains in the mitochondrial matrix. The mechanism of action of this chaperonin is outlined in some detail in the introduction to chapter 7). Cheng *et al.* (1989) using the yeast temperature sensitive mutant *mif4*, which is deficient in the function of hsp60, demonstrated a role for this chaperonin in the folding of translocated mitochondrial proteins. Both *in vivo* and *in vitro* studies revealed that although precursor proteins could be successfully translocated into the mitochondrial matrix of the mutant strain the folding of these precursor to yield functional proteins could not be achieved. Hsp60 has been shown to require ATP in assisting the folding of polypeptides in mitochondria (Ostermann *et al.*, 1989).

A model has been proposed which suggests that hsp70 and hsp60 work together in the folding of mitochondrial proteins. It has been postulated that precursors are passed from hsp70 to hsp60 during the folding process (Neupert *et al.*, 1990; Langer *et al.*, 1992).

## 1.8 Aims of thesis

1) To study the unfolding of a number of mul tisubunit enzymes brought about by GdnHCl, using c.d., fluorescence and enzyme activity measurements to monitor the

process. The proteins concerned included three translocated mitochondrial proteins (fumarase, NAD<sup>+</sup>-dependent isocitrate dehydrogenase, citrate synthase) and three bacterial proteins (Citrate synthases from *Thermoplasma acidophilum* and *Acinetobacter anitratum* and GroEL from *E. coli* ). Some of the properties of the proteins to be examined in this study are summarised below:

Protein studied	M <sub>r</sub>	Number of subunits	Number of tryptophans (per subunit)	High resolution structural data available
Fumarase	200 000	4	2	No
NAD <sup>+</sup> -Isocitrate dehydrogenase	316 000	8	1	No
Citrate synthases: pig heart	100 000	2	9	Yes
<i>Thermoplasma acidophilum</i>	85 880	2	4	Yes
<i>Acinetobacter anitratum</i>	284 000	6	4	No
GroEL	800 800	14	0	Yes

- 2) To investigate the extent to which the changes could be reversed by lowering the concentration of GdnHCl.
- 3) To compare the effects of lowering the concentration of GdnHCl by dialysis or dilution and the influence of other factors such as dithiothreitol, the presence of other proteins, temperature and ionic concentration of buffer on the regain of native properties.
- 4) To draw conclusions relating to the mechanism of protein folding and assembly *in vivo*.

## *Chapter two*

### **Materials and Methods**



## 2.1 Materials

The following materials were obtained from Sigma Chemical Co., Poole, Dorset, U.K:

Bovine Serum Albumin (BSA)  
Camphorsulphonic Acid (CSA)  
Coomassie Brilliant Blue G-250  
Dalton Mark VII-L (SDS-PAGE  $M_r$  markers)  
Dithiothreitol (DTT)  
Polyethylene Glycol (PEG)  
Sodium Chloride  
Sodium Dodecyl Sulphate (SDS)  
Sodium Phosphate  
N,N,N',N'-tetramethylethylene diamine (TEMED)  
Tris (hydroxymethyl) amino methane

Guanidinium Chloride (GdnHCl) of ultrapure grade was supplied by Gibco-BRL, Ltd., Paisley, Scotland, UK. Double-distilled water was used in all experiments.

## 2.2 Methods

### 2.2.1 Estimation of protein concentration

Protein concentrations were generally estimated for each enzyme either spectrophotometrically from the absorbance value at 280nm (using the published absorption coefficients of a 1mg/ml solution in a 1cm pathlength cuvette) or by the more sensitive Coomassie Blue binding assay. The latter method was developed by Sedmak and Grossberg (1977) and is outlined below:

A known volume of protein solution was diluted to 1ml with distilled water. 1ml of Coomassie blue reagent comprising 0.06% w/v Coomassie Brilliant Blue G-250 in 3% v/v perchloric acid was added and the absorbance of the resulting solution was

measured spectrophotometrically at 620nm and 465nm using distilled water as the blank. The absorbance ratio 620/465 was calculated after subtracting the absorbance ratio of 1ml distilled water plus 1ml Coomassie Blue reagent. A standard curve was constructed, using known amounts of bovine serum albumin between 0 and 50µg, from which the amount of unknown protein could be determined.

### 2.2.2 SDS polyacrylamide electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed in 12% acrylamide slab gels using the modified discontinuous system described by Laemmli (1970). Slab gels were prepared and run in vertical slab gel apparatus purchased from Bethesda Research Laboratories Inc. Solutions used in the preparation of the gels and protein samples are shown in Table 2.1. Samples were prepared by mixing with an equal volume of sample buffer and boiling for 3 min. A mixture (1:1) of 2-mercaptoethanol and 1% w/v bromophenol blue was added to the boiled samples at a concentration of 5% of the total sample volume. The samples were then loaded onto the gels using a Hamilton syringe. Electrophoresis was carried out at a constant current of 60mA until the bromophenol tracking dye reached the bottom of the separating gel (approx. 5 hours). Following electrophoresis the gels were stained with 0.1% Coomassie Brilliant Blue R250 for 1 hour and destained in 10% v/v acetic acid with several changes of destain solution.

Protein  $M_r$  values were determined from SDS PAGE by comparison with the relative migration of a set of Dalton reference proteins of known molecular weight. These marker proteins were as given below:

<b>Protein</b>	<b>Subunit <math>M_r</math></b>
Bovine serum albumin	66 000
Ovalbumin	45 000
Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle	36 000
Bovine carbonic anhydrase	29 000
Soybean trypsin inhibitor	20 000
$\alpha$ -lac_talbumin	14 000

**Table 2.1 Solutions used for SDS-polyacrylamide gels**

**Separating gel buffer:**

1.5M Tris  
0.4% SDD  
pH to 8.7 with HCl

**Stacking gel buffer:**

0.5M Tris  
0.4% SDS  
pH to 6.8 with HCl

**Sample buffer 2x conc:**

30ml 10% SDS  
0.96M Glycine  
0.5% SDS  
pH is 8.3 when diluted

**Reservoir buffer 5x conc:**

0.125M Tris  
12.5ml Stacking gel buffer  
10ml Glycerol  
pH to 6.8 with HCl

**Acrylamide stock solution:**

30% Acrylamide  
0.8% Bis-acrylamide

**Staining solution:**

0.1% Coomassie blue R250  
in acetic acid, methanol, H<sub>2</sub>O  
(2:5:5)

**de-staining solution:**

10% acetic acid

**Separating gel 12%:**

Separating gel buffer	5.0ml
Distilled H <sub>2</sub> O	7.0ml
Acrylamide stock solution	8.0ml
TEMED	15μl
10% Ammonium persulphate	70μl

**Stacking gel buffer 4%:**

Stacked gel buffer	2.5ml
Distilled H <sub>2</sub> O	6.1ml
Acrylamide stock solution	1.34ml
TEMED	10μl
10% Ammonium persulphate	50μl

**Sample preparation:**

Sample in H<sub>2</sub>O; Dilute 1:1 with Sample buffer 2x containing 2μl 2-mercaptoethanol per ml; Heat in boiling water bath for 3 min; Add 1μl 1:1 mixture of 1% bromophenol blue / 2-mercaptoethanol per 10μl boiled sample.

The marker proteins were used to produce a standard curve of the relative mobility of the proteins against the logarithm of molecular weight. All reference molecular weight proteins were purchased from Sigma.

### **2.2.3 Circular dichroism studies**

The circular dichroism (c.d.) spectra of each protein were recorded in a Jasco J-600 spectropolarimeter at the c.d. facility at the University of Stirling. Spectra in the far u.v. (260-190nm) were recorded in cylindrical quartz cells of pathlengths 0.02cm, 0.05cm, 0.1cm or 0.5cm and spectra in the near u.v. region (320-260nm) were recorded in cells of pathlengths 0.5cm or 1cm. When deciding which cell to use a number of factors were taken into consideration, notably, protein concentration, solvent transparency, buffers used, the addition of other chemicals, for example dithiothreitol or EDTA, and the signal to noise ratio. It is mandatory that the total absorbance (A) of the cell, solvent and protein sample is less than 1.0 unit otherwise the instrument is unable to record accurate c.d. measurements. In general it was found that cells of pathlength 0.05cm and 0.02cm gave good spectra below 200nm provided the protein concentration was sufficient enough to counteract the effects of noise encountered at such wavelengths. When the protein concentration was less it was necessary to use cells of a longer pathlength.

The c.d. instrument was calibrated at regular intervals using a 0.06% w/v aqueous solution of (+)-10-camphorsulphonic acid (CSA) which has a characteristic c.d. maximum at 290.5nm and a c.d. minimum at 192.5nm. A 0.06% solution of CSA should give a maximum ellipticity of 189 mdeg at 290.5nm and the ratio for the absolute intensities of the c.d. maximum and minimum peaks should be 2.0 or greater.

### **2.2.4 C.d spectra in the presence of GdnHCl**

Protein samples in the presence and absence of GdnHCl were prepared and allowed to incubate for 15 minutes at room temperature before c.d. spectra were recorded. All c.d. measurements were corrected for the contribution of GdnHCl and/or buffer using appropriate blanks.

### **2.2.5 Correction and analysis of c.d. data**

C.d. data were corrected for protein concentration, expressed as the molar concentration

of peptide bond units and cell pathlength by the mathematical data processing software of the IBM PS2 computer which operates the instrument. The corrected c.d. spectra were plotted as units of ellipticity ( $\theta$ , deg.cm<sup>2</sup> dmol.<sup>-1</sup>) as a function of wavelength (nm).

The analyses of c.d. data in terms of the estimation of secondary structure content were generally undertaken by applying the CONTIN procedure of Provencher and Glöckner, (1981), using data at 0.2nm intervals over the range 190-240nm. On occasions the methods of Chang *et al.* (1978), Seigel *et al.* (1980) and Chen *et al.* (1974) were employed to determine whether or not the estimates of secondary structure were comparable with the CONTIN procedure. A brief summary of each of these methods is given in Appendix 1.

#### **2.2.6 Fluorescence studies**

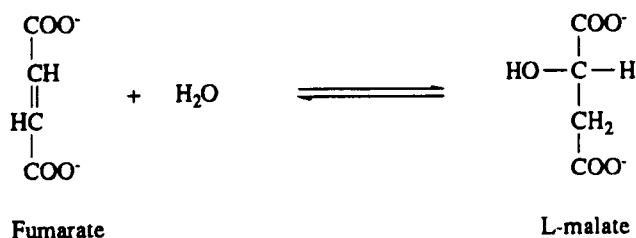
The fluorescence studies on fumarase were recorded on a Perkin-Elmer MPF 3A spectrofluorimeter. The studies on isocitrate dehydrogenase, citrate synthase, malate dehydrogenase and GroEL were recorded on a Perkin-Elmer LS50 spectrofluorimeter. Samples were measured at 20 °C, unless otherwise indicated, in quartz cells of pathlength 1cm. The excitation wavelength was typically 290nm and emission spectra were recorded between 300 and 400nm using appropriate slit widths depending on the protein concentration. Protein samples were incubated in buffer in the presence or absence of GdnHCl before the spectra were recorded. All measurements were corrected for the contribution of GdnHCl and/or buffer using appropriate blanks.

*Chapter three*

**The Unfolding and Refolding of Pig Heart Fumarase**

### 3.1 Introduction

Fumarase (fumarate hydratase EC 4.2.1.2.) is widely distributed in animals, plants and microorganisms due to its essential role in the tricarboxylic acid- cycle where it catalyses the reversible hydration of fumarate to L-malate as outlined below:



The earliest experiments describing the action of fumarase were carried out in 1910 when Battelli and Stern demonstrated that beef liver homogenates converted succinate to malate. Later Einbeck (1919) reported that the conversion of succinate to malate involved the oxidation of succinate to fumarate followed by the hydration of fumarate to malate. Battelli and Stern (1921) named the enzyme involved in the latter step of this reaction fumarase (for review describing these early experiments see Hill and Teipel, 1971). The characterisation of fumarase became more credible following its crystallization from pig heart muscle by Massey in 1952 yielding purer enzyme. Over the years various groups have modified the purification procedure to give an improved yield of fumarase (notably Frieden *et al.*, 1954; Kanarek and Hill, 1964; Beckmans and Kanarek, 1977).

A great deal is now known about the structure of fumarase. Kanarek *et al.* (1964) first reported that the enzyme from porcine heart was a tetrameric enzyme of  $M_r$  194 000 containing a total of about 1760 residues per molecule. Their studies showed that fumarase contains 12 cysteine residues but is devoid of disulphide bonds. Kanarek *et al.* concluded from these results that the four subunits must be linked in the native

enzyme through non-covalent bonds. Sacchettini *et al.* (1986) have since confirmed these findings although they have suggested, from sequencing studies, that the  $M_r$  of native fumarase is approximately 200 000 and that each identical subunit contains 466 amino acids.

The fumarase enzymes from various species have been shown to possess a very significant degree of sequence identity as indicated in Table 3.1. In addition several highly conserved regions are observed when each of the amino acid sequences are compared. These regions correspond to Gln-98 to Asn-115, Lys-186 to Gly-203, Gly-231 to Asn-240, Lys-293 to Gly-308 and Gly-320 to Pro-330. Sacchettini *et al.* (1988) have speculated that the amino acids located at these sites may well function at the active site of fumarase. Medical studies have revealed that a deficiency in the enzyme fumarase is a rare autosomal recessive disorder which causes severe neurological impairment. Recent evidence has shown that a mutation which leads to a glutamate to glutamine transition in a highly conserved region of the enzyme can cause this deficiency (Bourgeron, 1993). In addition, a mutation which results in an alanine to threonine transition at position 265 of the human amino acid sequence of fumarase has also been reported to cause this disorder.

**Table 3.1** A comparison of the sequence identity of pig heart fumarase with fumarases from various sources

Fumarase	% Sequence identity <i>c.f.</i> pig heart fumarase
human liver	96
yeast	67
<i>E-coli</i> fum C gene product	61
<i>Bacillus subtilis</i>	58



Fumarase is believed to occur in the matrix of the mitochondria although some evidence has suggested that it may bind to the mitochondrial inner membrane (Srere, 1982). The mitochondrial enzyme is synthesised as a precursor in the cytosol before being imported into the mitochondrion. A number of laboratories have reported the existence of two isoenzymes of fumarase which are located in the mitochondria and cytosol respectively. Such heterogeneity has thus far been observed in fumarases from rat, pig and human liver as well as from yeast. In addition there is strong evidence to suggest that the isoenzymes have almost identical amino acid sequences and could possibly be encoded by a single gene (Ono *et al.*, 1985; O'Hare and Doonan, 1985; Kinsella and Doonan, 1986; Wu and Tzagoloff, 1987; Sacchettini *et al.*, 1988; Suzuki *et al.*, 1989).

At present there is little information on the secondary or tertiary structure of fumarase. Optical-rotatory-dispersion measurements (Kanarek and Hill, 1964) and the Fasman secondary structure prediction program, based on the amino acid sequence of proteins (Chou and Fasman, 1978; Sacchettini *et al.*, 1988) have indicated that fumarase contains a large proportion of  $\alpha$ -helix. X-ray crystallographic studies on fumarase have yet to provide high resolution structural data.

The folding properties of fumarase were first examined over twenty years ago (Teipel and Koshland, 1971; Teipel and Hill, 1971). These workers reported that after denaturation, fumarase regained some activity following dilution of guanidinium chloride (GdnHCl). In view of the improved purity and stability of fumarase using modern purification techniques and the difficulty in refolding other mitochondrial matrix proteins (West and Price, 1988, 1990; West *et al.*, 1990) it was considered of interest to investigate further the structural properties of fumarase and to re-examine the unfolding and refolding of the enzyme in more detail.

## **3.2 Materials and Methods**

### **3.2.1 Materials**

Pig heart fumarase, prepared by affinity chromatography (Beeckmans and Kanarek, 1977) was purchased as an ammonium sulphate suspension from Sigma Chemical Co. Malic acid was purchased from Sigma. Sepharose 4B gel separation column (1.8 x 10cm) was purchased from Pharmacia.

Other chemicals which were routinely used are listed in chapter 2.

### **3.2.2 Methods**

#### **3.2.2.1 Determination of protein concentration**

The protein concentration of fumarase was determined by the Coomassie Blue binding method (Sedmak and Grossberg, 1977) outlined in Section 2.2.1. Amino acid analysis (carried out at the WELMET Protein Characterisation Facility at the University of Edinburgh) using the published amino acid composition data (Beeckmans and Kanarek, 1977) was used to confirm the results obtained by the dye-binding method. Preliminary experiments using spectrophotometric determination at 280nm gave unreliable results due to light scattering. These problems have previously been noted by Kanarek and Hill (1964).

The concentration of fumarase was increased, when necessary, either by centrifugation of a 90%  $(\text{NH}_4)_2\text{SO}_4$  suspension for 20 min at 20000g) and redissolving in a smaller volume of 50mM sodium phosphate buffer, pH 7.3 or by dialysing the enzyme against a 20% (w/v) solution of poly (ethylene glycol  $M_r$  approximately 8000) in 50mM sodium phosphate buffer, pH 7.3.

#### **3.2.2.2 Assay of fumarase activity**

Enzyme activity was assayed by monitoring the conversion of L-malate to fumarate spectrophotometrically at 250nm. Fumarase was added to a 1cm pathlength quartz cell containing 1ml of 50mM-L-malate in 50mM-sodium phosphate buffer, pH 7.3 and the rate of activity was recorded at 25 °C.

### **3.2.2.3 Denaturation of fumarase in the presence of GdnHCl**

The denaturation of fumarase by GdnHCl was monitored by activity studies, circular dichroism and fluorescence.

### **3.2.2.4 Loss of catalytic activity**

Fumarase samples (30µg/ml) were incubated for 15 minutes at 20 °C in 50mM-sodium phosphate, pH 7.3 containing 1mM dithiothreitol and increasing concentrations of GdnHCl. 20µl aliquots were removed and added to the assay mix which contained the same concentration of GdnHCl as the corresponding incubation mix. The catalytic activity was monitored as described previously.

### **3.2.2.5 Changes in secondary and tertiary structure**

The effect of GdnHCl on the secondary and tertiary structure of fumarase was monitored by circular dichroism and fluorescence studies as outlined in sections 2.2.3 and 2.2.4 respectively.

### **3.2.2.6 Changes in quaternary structure**

The quaternary structure of fumarase in the absence and presence of GdnHCl was examined by gel filtration on a Sepharose 4B column (1.8cm x 10cm). The void volume of the column was determined using blue dextran ( $M_r$  2 x 10<sup>6</sup>). Samples (1ml) of enzyme (30µg/ml) were applied to the column and eluted with 50mM-sodium phosphate buffer, pH 7.3. Fractions (1ml) were collected and the elution of protein was monitored by fluorescence using excitation and emission wavelengths of 290 and 325nm respectively. Proteins of known molecular weight were used to calibrate the column (see section 3.3.2.6).

### **3.2.2.7 Reactivation of fumarase**

Following incubation of fumarase for 15 minutes in GdnHCl the reactivation of enzyme was attempted either by dilution into buffer or by dialysis of the denaturant using 50mM sodium phosphate pH 7.3 in the presence or absence of dithiothreitol. The residual concentrations of GdnHCl following dilution or dialysis were shown by refractive index measurements to be 0.02M and less than 0.01M respectively. Activity studies, gel filtration, fluorescence and c.d. were used to assess the degree of refolding.

### **3.3 Results and Discussion**

#### **3.3.1 Secondary structure of native fumarase**

The far u.v. circular dichroism spectrum of fumarase is shown in Figure 3.1. The spectrum of the native enzyme shows a negative ellipticity over the wavelength range 240-202nm. The curve exemplifies the characteristics of a classical  $\alpha$ -helical protein with its negative double maxima at 222nm and 208nm and the move towards positive ellipticity at approximately 202nm. The positive elliptical maximum below 195nm is often considered the “fingerprint” region in the determination of the  $\alpha$ -helical content of a given protein for which reason it is necessary to obtain spectra in conditions which give a high signal to noise ratio as explained in section 2.2.3. The percentages of  $\alpha$ -helix and  $\beta$ -sheet were estimated as  $57\pm 1.6$  and  $26\pm 2.2$  respectively, using the CONTIN secondary structure analysis program (Provencher and Glöckner, 1981) over the range 190-240nm. Earlier work by Kanarek and Hill (1964) estimated an  $\alpha$ -helical content of around 50% based on optical-rotatory-dispersion measurements.

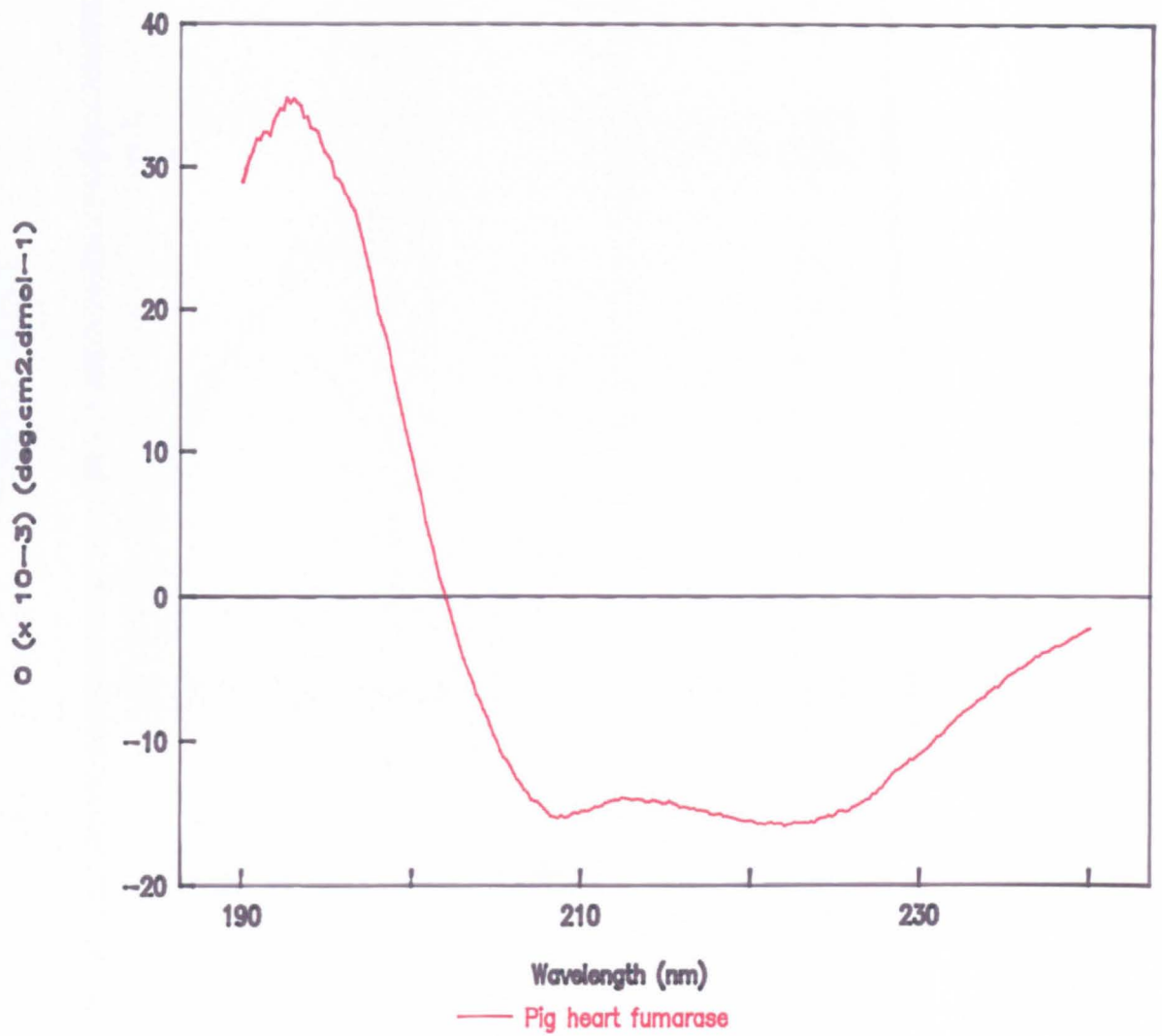
#### **3.3.2 Unfolding of fumarase by GdnHCl**

##### **3.3.2.1 Changes in catalytic activity**

The activity of fumarase in the presence of GdnHCl is shown in Figure 3.2. It is evident from these data that the presence of denaturant, even at very low concentrations, causes a considerable loss in the catalytic activity of fumarase. For example in the presence of 0.1M GdnHCl the enzyme has retained only 45% of its original activity indicating that the active site of fumarase is highly sensitive to the perturbations caused by low concentrations of denaturant.

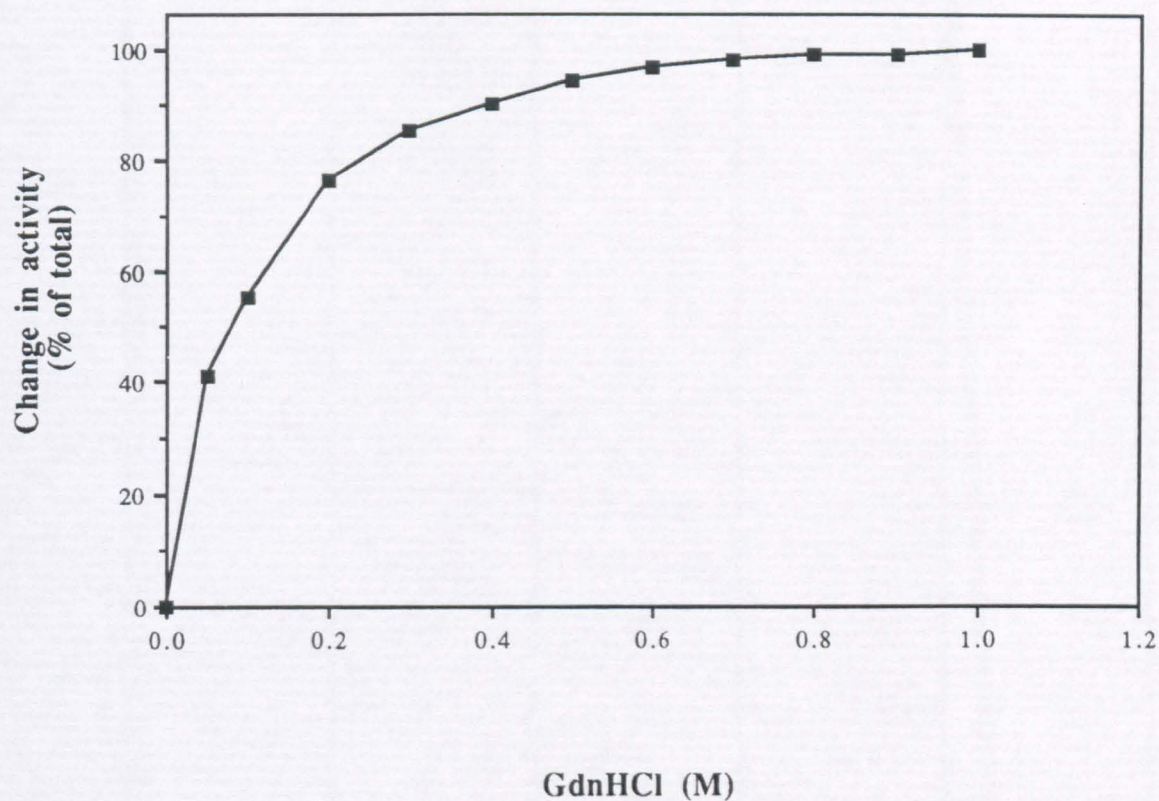
##### **3.3.2.2 Changes in fluorescence**

The fluorescence emission spectra of fumarase in the presence and absence of 4M GdnHCl are shown in Figure 3.3. When excited at 290nm the maximum emission of the native enzyme was observed at 325nm. This values suggests that the tryptophan residues in native fumarase are not exposed to the solvent but are buried in the core of the protein. In the presence of 4M GdnHCl there was a 65% reduction in the intensity of emission and the wavelength maximum was shifted towards the red resulting in a peak at 353nm, a value close to that characteristic of tryptophan side chains exposed to the solvent. Figure 3.4 shows the effect of increasing concentrations of GdnHCl on the



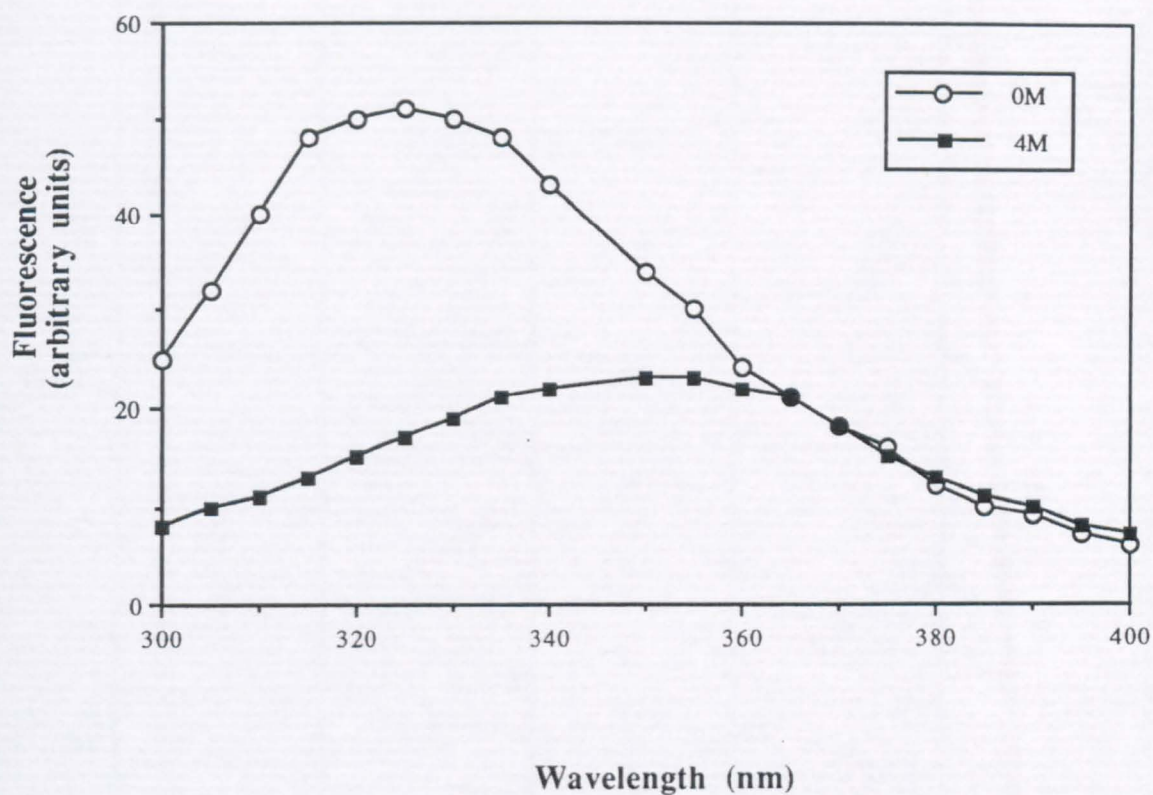
**Figure 3.1** Far-u.v. c.d. spectrum of fumarase (50 $\mu$ g/ml)

The spectrum was recorded at 20 °C in 50mM sodium phosphate buffer, pH 7.3.



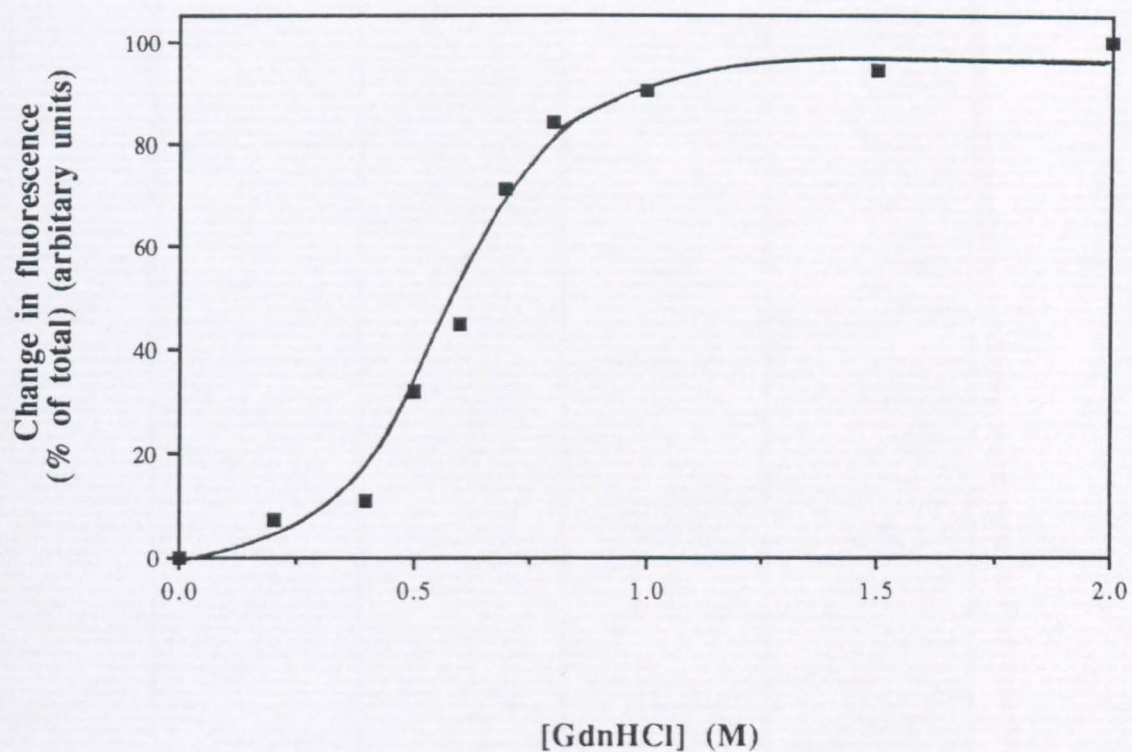
**Figure 3.2** Activity of fumarase as a function of concentration of GdnHCl

Enzyme (30 $\mu$ g/ml) was incubated for 15min at 20°C in 50mM sodium phosphate buffer (pH 7.3) containing 1mM-dithiothreitol in the presence of increasing concentrations of GdnHCl. The changes in activity are expressed relative to the total change observed between 0 and 4M GdnHCl.



**Figure 3.3** Fluorescence emission of fumarase in the absence and presence of 4M GdnHCl

Enzyme (23 $\mu$ g/ml) was incubated for 15min at 20°C in 50mM sodium phosphate buffer (pH 7.3) containing 1mM-dithiothreitol in the presence of the indicated concentration of GdnHCl.



**Figure 3.4** Changes in the fluorescence emission of fumarase at 325nm as a function of concentration of GdnHCl

Enzyme (23 $\mu$ g/ml) was incubated for 15min at 20°C in 50mM sodium phosphate buffer (pH 7.3) containing 1mM-dithiothreitol in the presence of the indicated concentration of GdnHCl.



relative fluorescence of fumarase at 325nm. It can be seen from these data that the intensity of the emission maximum decreases with increasing concentration of denaturant and the consequential loss of tertiary structure. The midpoint of the transition in fluorescence intensity was observed at approximately 0.65M GdnHCl.

#### **3.3.2.3 Changes in circular dichroism**

The circular dichroism spectra of fumarase in the presence and absence of 4M GdnHCl are shown in Figure 3.5. for comparison. The spectrum of fumarase in 4M GdnHCl represents protein in an unordered or random conformation as described by Chang *et al.* (1978). Figure 3.6 shows the sequential denaturation of fumarase as a function of increasing concentrations of GdnHCl. The change in ellipticity at 225nm reflects the change in helical content of the enzyme (Chen *et al.*, 1974). From these data it can be seen that fumarase exhibits a considerable loss of helical structure between 0M and 2M GdnHCl and at 4M GdnHCl the unfolding of enzyme is essentially complete. The midpoint of the transition between native and unfolded fumarase in terms of  $\theta_{225}$  occurs at 1.1M GdnHCl.

#### **3.3.2.4 Unfolding in the presence and absence of DTT**

The unfolding of fumarase in the presence and absence of 1mM DTT, as monitored by activity studies, fluorescence and circular dichroism was also examined to establish whether or not the presence of the reducing agent affected the unfolding process. It was found that it did not affect the activity of fumarase under these conditions. Examination of the fluorescence properties of the enzyme under denaturing conditions (Figure 3.7) indicated that the presence of DTT seems to increase the degree of unfolding in the range from 0.7 to 0.9M GdnHCl. By contrast there was a greater degree of unfolding in the absence of DTT in this range of GdnHCl concentrations as judged by c.d (Figure 3.8). Overall the differences were relatively small indicating that the presence of DTT has little effect on the GdnHCl-induced unfolding of fumarase.

#### **3.3.2.5 Time dependence of unfolding**

As noted in section 2.2.4, proteins were routinely incubated in denaturant for a period of 15 minutes. The unfolding of fumarase in the presence of certain concentrations of GdnHCl, however, was time-dependent in that further unfolding was observed after the

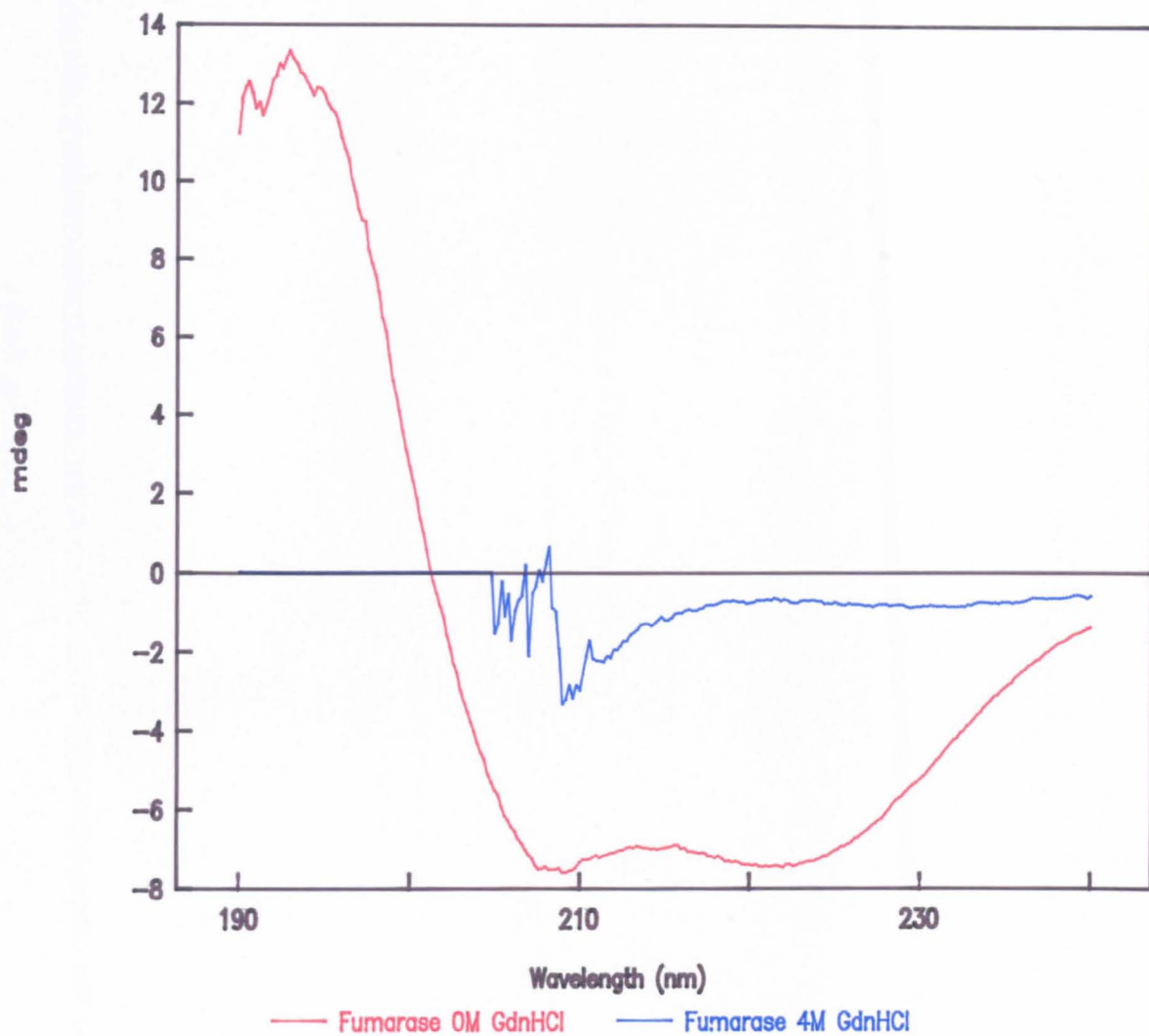
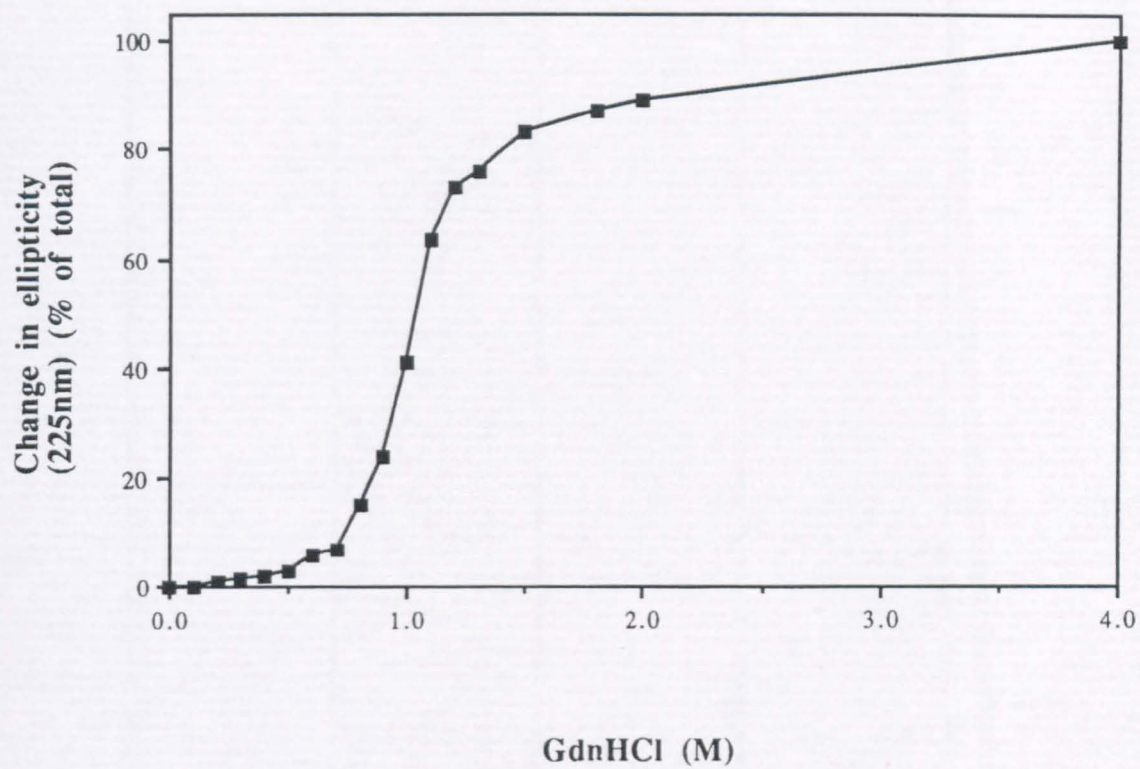


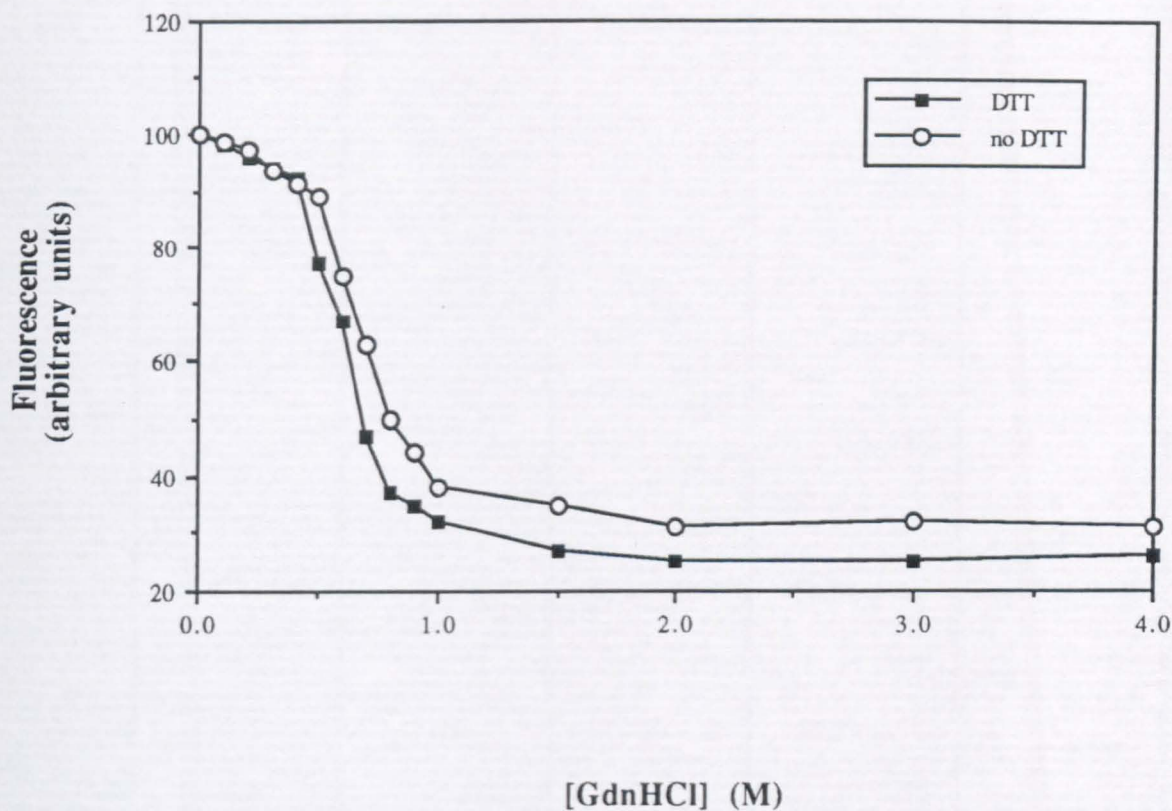
Figure 3.5 The far u.v. c.d.spectra of fumarase (50 $\mu$ g/ml) in the presence and absence of 6M GdnHCl

The spectra were recorded at 20 °C in 50mM-sodium phosphate buffer, pH 7.3.



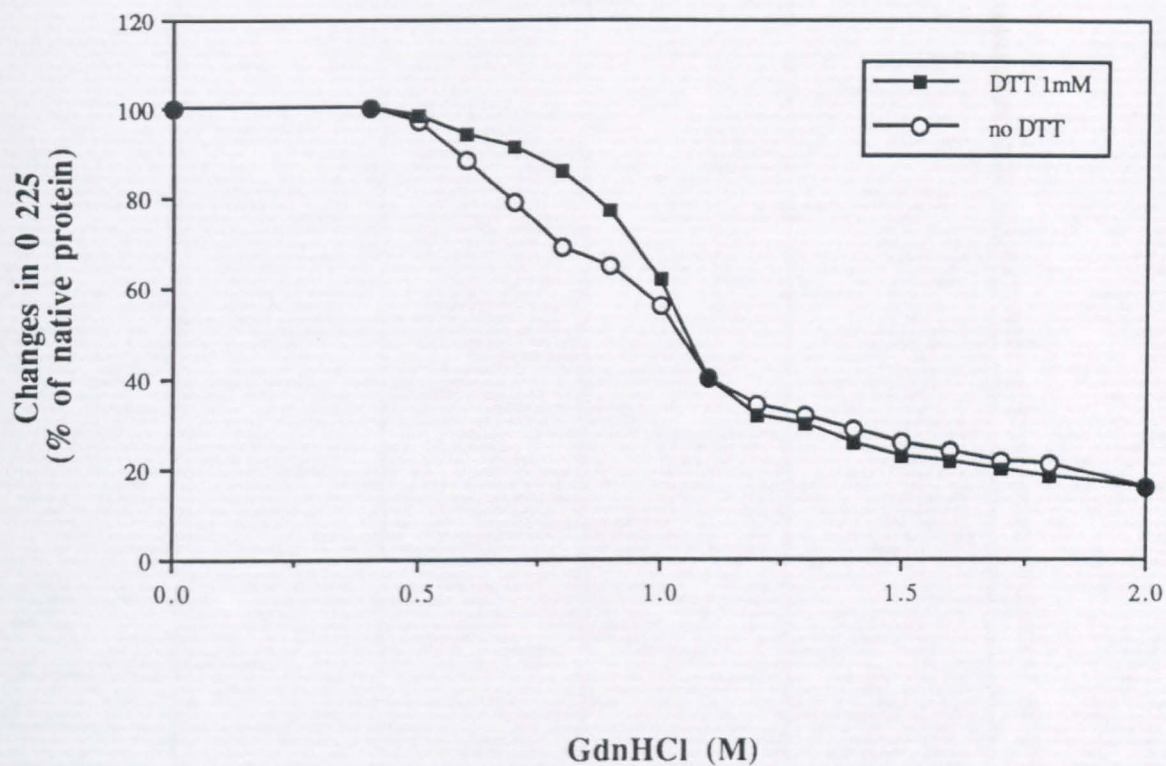
**Figure 3.6** Changes in ellipticity at 225nm as a function of concentration of GdnHCl

Changes in  $\theta_{225}$  are expressed relative to the total change observed between 0 and 4M GdnHCl.



**Figure 3.7** Changes in the fluorescence emission of fumarase at 325nm in the absence and presence of dithiothreitol, as function of concentration of GdnHCl

Fumarase samples (20 $\mu$ g/ml) were incubated for 15 min at 20°C in 50mm sodium phosphate buffer (pH 7.3) containing the indicated concentrations of GdnHCl in the absence and presence of dithiothreitol.



**Figure 3.8** Changes in ellipticity at 225nm of fumarase in the absence and presence of dithiothreitol, as function of concentration of GdnHCl

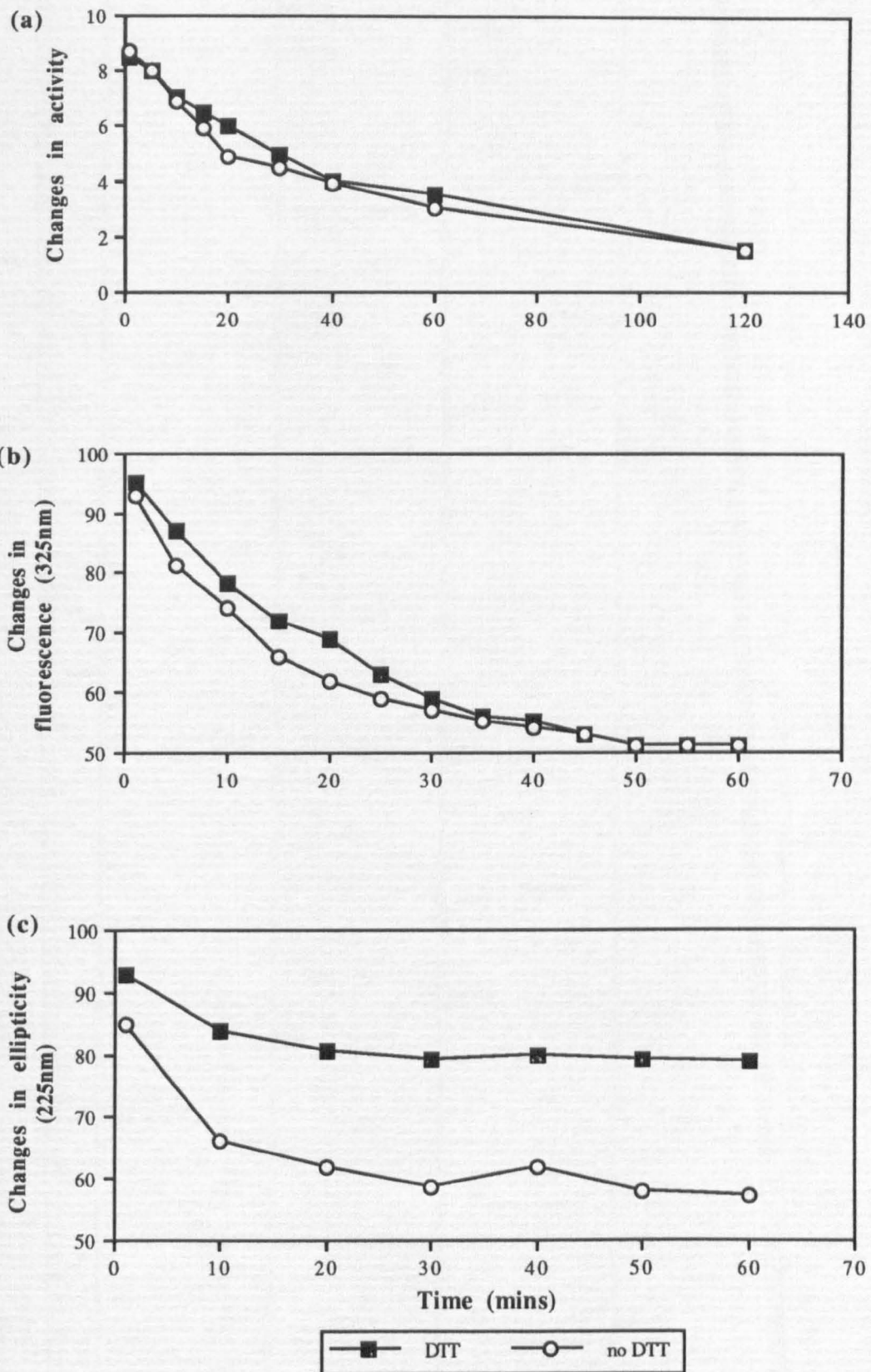
Fumarase samples (20 $\mu$ g/ml) were incubated for 15 min at 20°C in 50mm sodium phosphate buffer (pH 7.3) containing the indicated concentrations of GdnHCl in the absence and presence of dithiothreitol.

15 min incubation period. This time-dependency was not observed at concentrations of GdnHCl of 0.4M and below or 1M and above where unfolding was essentially complete after approximately 1min. It was found that the concentrations of GdnHCl at which time-dependency was observed were different for each of the parameters measured. In the case of the activity and fluorescence studies these changes were noticed over the ranges 0.5M to 0.7M and 0.5M to 0.9M GdnHCl respectively. The data relating to changes in activity and fluorescence in 0.5M GdnHCl as a function of time are shown in Figures 3.9a and 3.9b respectively. The changes in secondary structure occurred between 0.7M and 0.8M GdnHCl, inclusive. It was also noted that in the absence of dithiothreitol there was a marked difference in the length of time taken for fumarase to reach the "end point" of unfolding at both 0.7M and 0.8M GdnHCl. Figure 3.9c shows the time-dependency of unfolding of fumarase at 0.8M GdnHCl under reducing and non-reducing conditions. These effects in the presence and absence of dithiothreitol were also reflected in the fluorescence studies, however the reducing agent did not significantly influence the rate of inactivation of fumarase in the "time-dependent" range described above.

#### **3.3.2.6 Changes in quaternary structure**

Gel filtration on Sepharose 4B was used to examine the quaternary structure of fumarase under denaturing and non-denaturing conditions. It was found that the elution volume of native enzyme (in the absence of denaturant) was 18ml. In the presence of GdnHCl the elution volume of the enzyme was found to be different at each of the concentrations of denaturant examined. For example in 0.5M GdnHCl the elution volume of fumarase increased to 23ml while in 2M GdnHCl the fluorescence optimum of eluted protein was found in the sample eluted at 19ml. Proteins of known molecular weight were also examined on the column, including trypsinogen, yeast phosphoglycerate mutase, bovine serum albumin, aldolase, catalase and glutamate dehydrogenase (purchased from Sigma (section 2.2.2 for  $M_r$  values). The elution volume of yeast phosphoglycerate mutase ( $M_r$  45 000), was 23ml which corresponded to that of fumarase in the presence of 0.5M GdnHCl suggesting that at this concentration of denaturant fumarase is dissociated into subunits.

The results obtained for fumarase on gel filtration at concentrations of GdnHCl above



**Figure 3.9 Time dependence of unfolding of fumarase by intermediate concentrations of GdnHCl**

Figures 3.9 (a) and (b) refer to changes in activity and fluorescence (325nm) of fumarase respectively in buffer containing 0.5M GdnHCl. Figure 3.9c refers to changes in ellipticity (225nm) of fumarase in buffer containing 0.8M GdnHCl. In each case enzyme (20-30µg/ml) was examined in the absence and presence of 1mM dithiothreitol (DTT). All data are expressed relative to the native enzyme which corresponds to a value of 100.

0.5M GdnHCl were difficult to interpret. As noted above the elution volume of fumarase in 2M GdnHCl was 19ml, a value close to that obtained for the native protein. It is probable that in this concentration of GdnHCl the subunits of the protein are in an unfolded state and therefore of higher Stokes radius than the monomer thus leading to elution at a lower volume.

### **3.3.3 Refolding of fumarase**

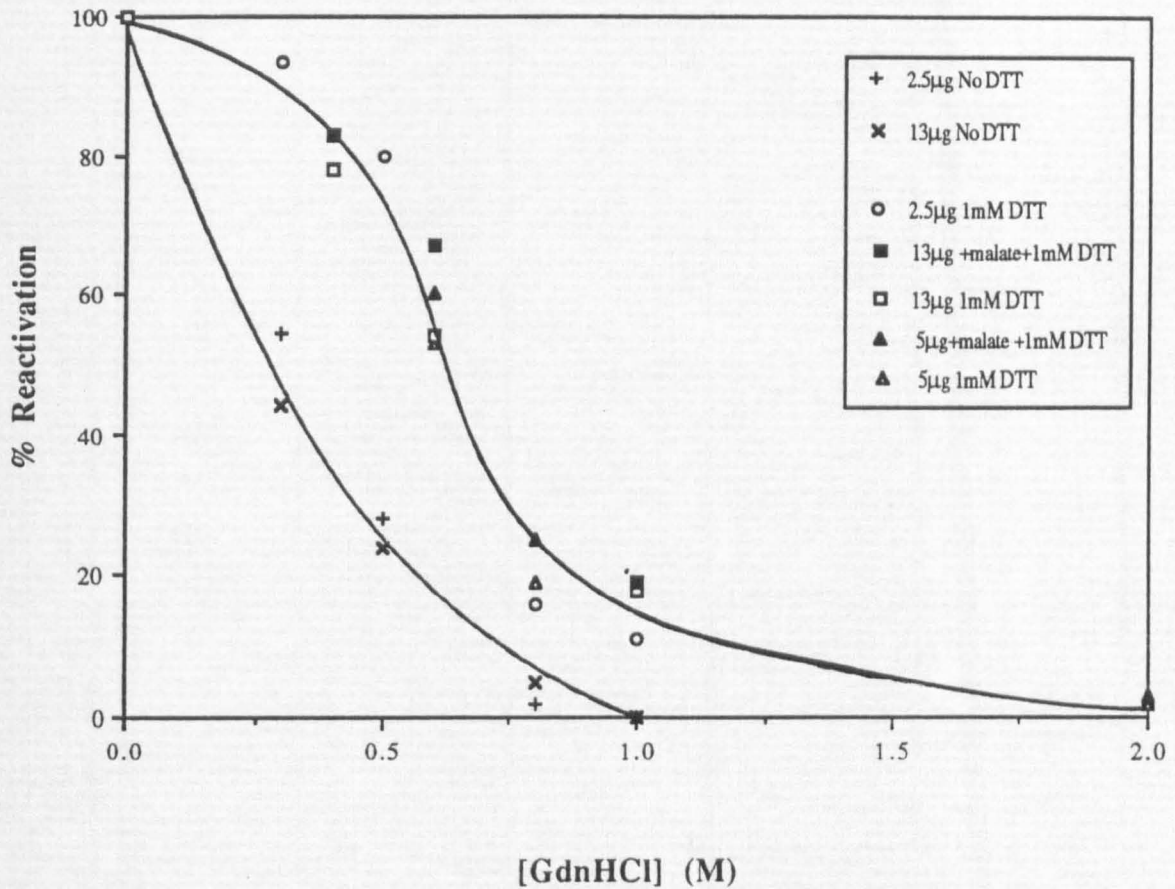
The ability of denatured fumarase to refold was examined following the removal of denaturant, either by a 60-fold dilution into 50mM-sodium phosphate pH 7.3 or by dialysis against 200 volumes of the same buffer. Activity studies, gel filtration, fluorescence studies and circular dichroism were employed to assess the extent of refolding.

#### **3.3.3.1 Reactivation of fumarase by dilution**

The results of the attempted reactivation of fumarase following incubation for 15min in increasing concentrations of GdnHCl and subsequent dilution (60-fold) are shown in Figure 3.10. The effects of enzyme concentration and including dithiothreitol or malate in the reactivation mix were also examined. It was found that fumarase was unable to regain activity when the initial concentration of denaturant was greater than 1M. In addition the reactivation of fumarase in the absence of dithiothreitol is lower than that observed in the presence of reducing agent or the substrate malate. Varying the enzyme concentration, between 1 and 17 $\mu$ g/ml, and the time of incubation in the dilution buffer (from 1h to 48h) did not affect the extent of reactivation. Lowering the concentration of denaturant by dilution is a rapid process, complete within about 5sec, compared with dialysis. An experiment was therefore set up which examined the effect of slowing down the rate of addition of the reactivation mix to the denatured enzyme using 50 $\mu$ l aliquots over a period of 3min. The results obtained by this method did not differ from those obtained following the rapid dilution procedure.

The low concentration of fumarase obtained following the dilution procedure unfortunately precluded the examination of the structural properties and the extent of regain of native conformation by gel filtration, fluorescence or circular dichroism. The poor regain of activity suggests that the lowering of the concentration of denaturant by





**Figure 3.10** Reactivation of fumarase following dilution of GdnHCl

Enzyme was incubated for 15 min at 20°C in 50mM sodium phosphate buffer (pH 7.3) containing the indicated concentrations of GdnHCl and dithiothreitol. GdnHCl was then diluted to give a residual concentration of 0.02M. In each case reactivation is expressed relative to a control sample incubated in the absence of GdnHCl.

dilution is not able to reverse the perturbations caused at the active site during incubation in GdnHCl at concentrations greater than about 0.5M GdnHCl.

#### **3.3.3.2 Reactivation by dialysis**

The effects of using the dialysis procedure to remove GdnHCl from denatured fumarase are shown in Table 3.2. It was found that provided dithiothreitol, at a concentration of at least 1mM, was present in the dialysis buffer, a substantial proportion of activity of fumarase could be regained. The values obtained for the percentage reactivation in the presence of reducing agent were calculated to be 62% and 48% (of the activity of native enzyme) following initial incubations in 1.5M and 4M GdnHCl respectively, and subsequent dialysis.

#### **3.3.3.3 Regain of quaternary structure**

Following dialysis the elution volume of fumarase (following gel filtration), which had been previously incubated in 4M GdnHCl was 18ml showing that the regain of secondary structure has resulted in a quaternary structure similar to that of the native enzyme (Figure 3.11). In the absence of reducing agent the elution volume of fumarase was found to be maximal at 23ml which indicates that following dialysis the enzyme is in a monomeric state.

#### **3.3.3.4 Regain of tertiary structure**

From Figure 3.12 and Table 3.2 it can be seen that following the dialysis procedure against buffer containing 1mM dithiothreitol the regain of tertiary structure of fumarase after initial incubation in the presence of 1.5M and 4M GdnHCl was found to be 94% and 79% respectively. The emission maxima which were red-shifted in the presence of GdnHCl were also restored to 325nm following dialysis in the presence of reducing agent. When denatured fumarase was dialysed against buffer devoid of dithiothreitol the ability of the enzyme to adopt tertiary structure similar to the native conformation was found to be markedly reduced. The emission maximum was shifted to the red compared with that of native fumarase indicating that following dialysis enzyme which has been pre-incubated in GdnHCl adopts a less “compact” tertiary structure which favours the exposure of tryptophan residues to a greater extent than found in the native enzyme.

**Table 3.2 Refolding of fumarase after denaturation in GdnHCl**

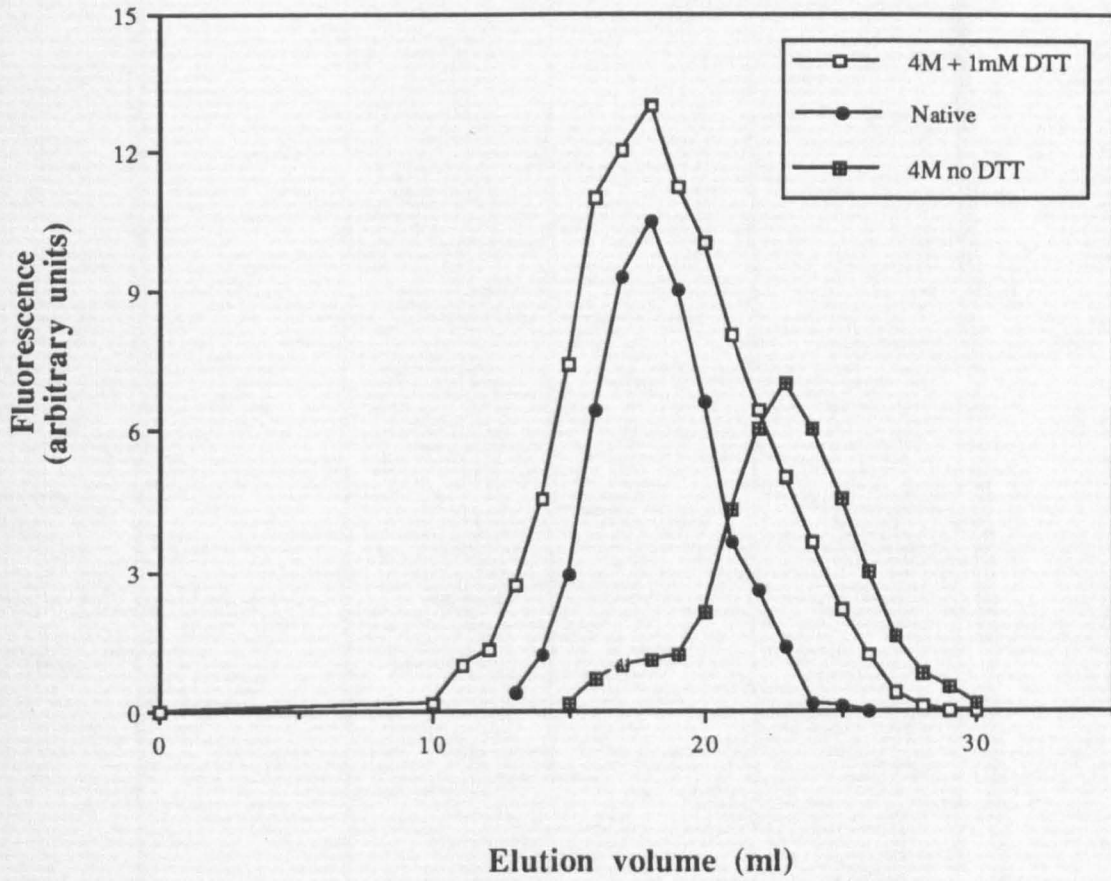
Sample	Activity†	F <sub>325</sub> †	Maximum† Emission	[θ] <sub>225</sub> †
Native enzyme	100	100	325	100
<b>Enzyme+1.5M GdnHCl + 1mM DTT*</b>				
<i>Before dialysis</i>	0	28	345	22
<i>After dialysis</i>	62	94	325	98
<b>Enzyme+1.5M GdnHCl</b>				
<i>Before dialysis</i>	0	28	345	22
<i>After dialysis</i>	3	72	333	96
<b>Enzyme + 4M GdnHCl + 1mM DTT*</b>				
<i>Before dialysis</i>	0	25	353	8
<i>After dialysis</i>	48	79	325	95
<b>Enzyme + 4M GdnHCl</b>				
<i>Before dialysis</i>	0	25	353	8
<i>After dialysis</i>	1	67	335	96

notes:

† - Relative

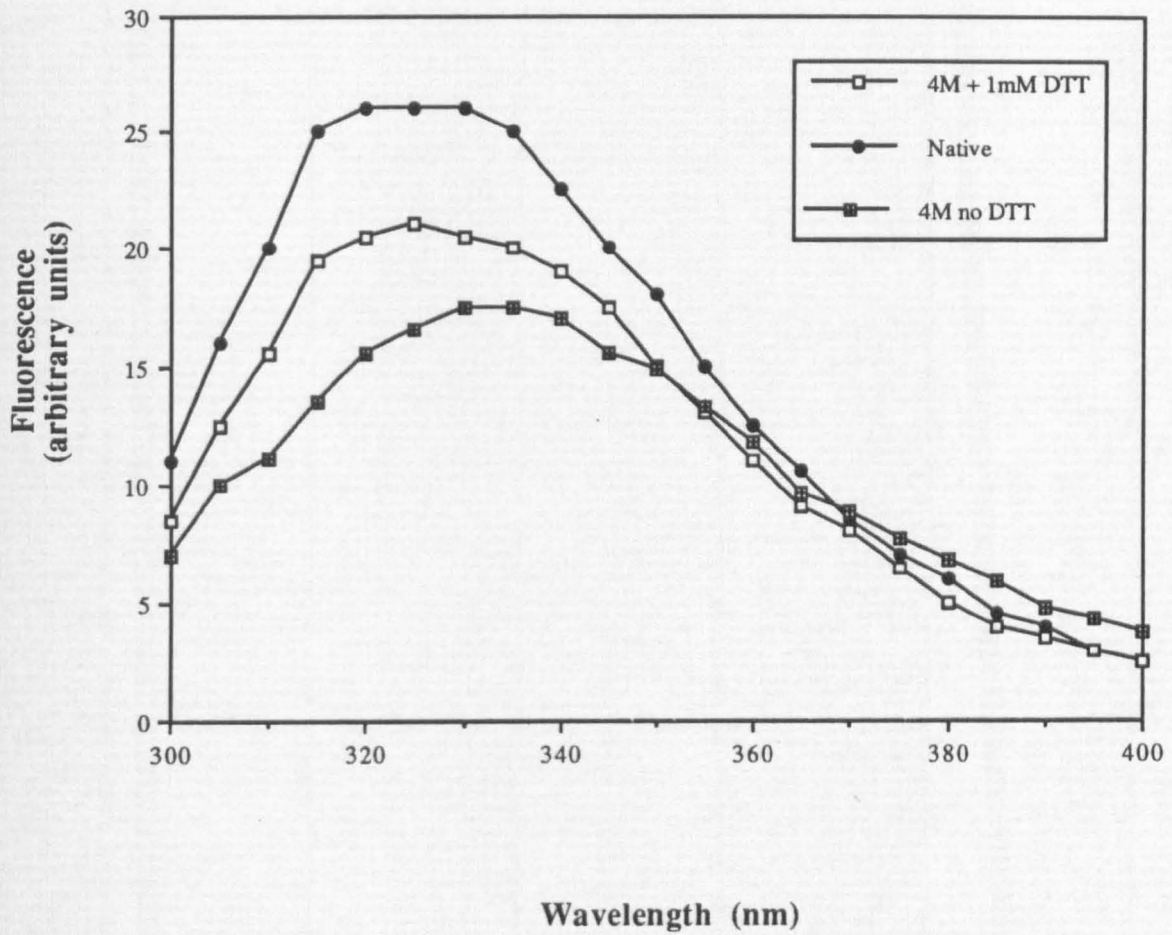
\* - 1mM dithiothreitol (DTT) was included in both the unfolding incubation mixture and the buffer against which the sample was dialysed.

Denaturation was performed by incubating the enzyme for 15 min at 20 °C with the stated concentration of GdnHCl and dithiothreitol. Dialysis was performed at 4 °C for 18 hours against 500 volumes of 50mM sodium phosphate buffer, pH 7.3. The final concentration of enzyme was 30 µg/ml. Values shown are the means of duplicate measurements with less than 10% variation in the values of activity, fluorescence and molar ellipticity. The precision of the emission maximum is estimated at ± 1nm.



**Figure 3.11** Fluorescence emission at 325nm of native and refolded fumarase following  $g\text{-}1$  filtration

Enzyme ( $30\mu\text{g/ml}$ ) was denatured in 4M GdnHCl and refolded in the absence and presence of 1mM dithiothreitol.



**Figure 3.12** Fluorescence emission of native and refolded fumarase following dilution of GdnHCl

Enzyme (25 $\mu$ g/ml) was incubated in 4M GdnHCl for 15 min at 20°C and then diluted with buffer in the absence and presence on 1mM dithiothreitol.

### **3.3.3.5 Regain of secondary structure**

Figure 3.13 shows the c.d. spectra of fumarase samples which had been incubated in 4M GdnHCl and dialysed in the presence and absence of reducing agent. The regain of native-like secondary structure following denaturation of fumarase and subsequent dialysis was found to be at least 90% complete regardless of the presence of dithiothreitol in the dialysis buffer (see also Table 3.2). In the presence of reducing agent, however, the regain of secondary structure was found to be almost identical to that of the native protein. It can be assumed from these results that the formation of secondary structure occurs prior to the step/s on the folding pathway in which the protein experiences difficulty in attaining its native conformation. It has been shown that the formation of secondary structure is an early step in the folding pathway which precedes that of 'compact intermediate' and the attainment of functional conformation (Jaenicke, 1987).

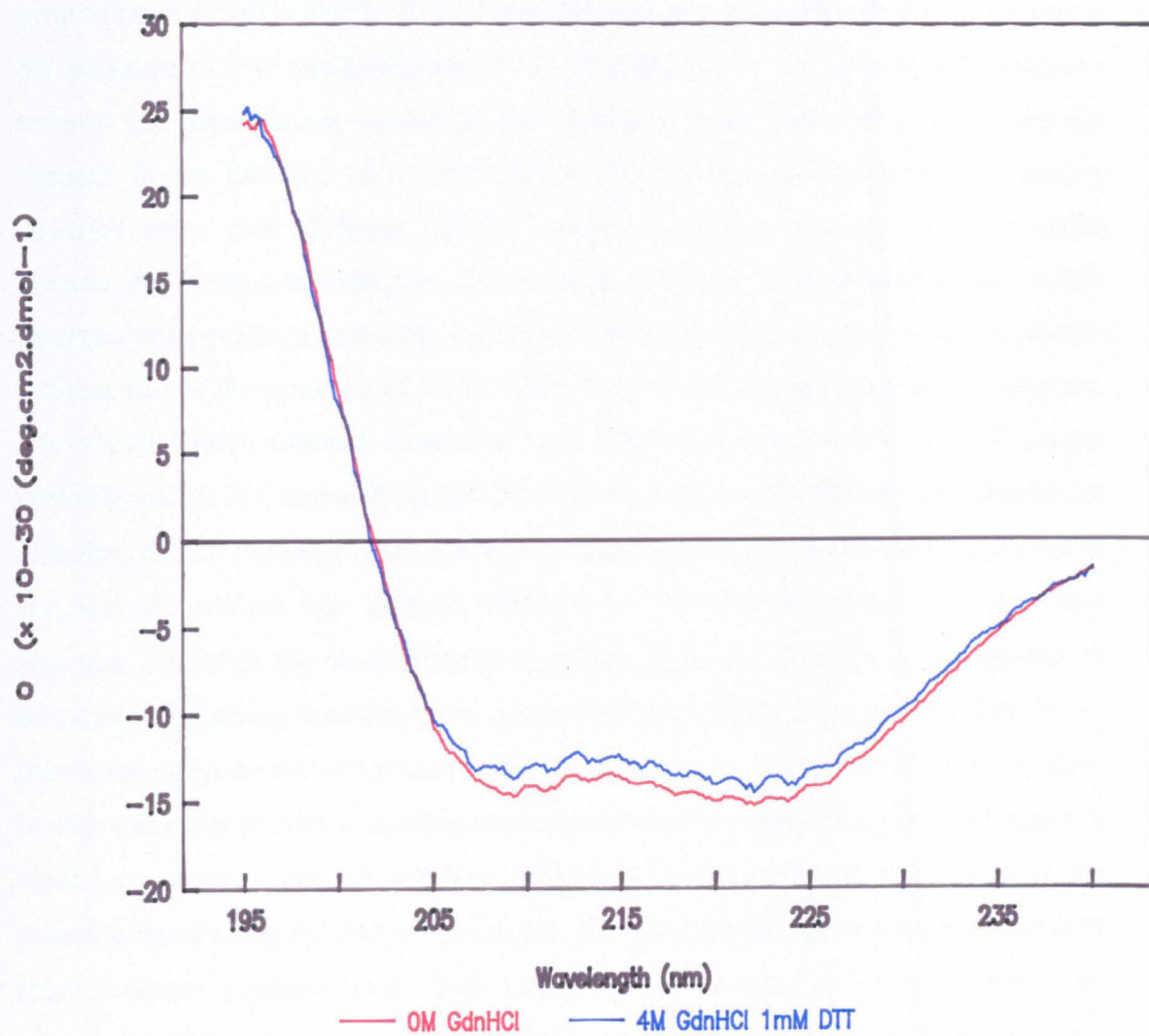


Figure 3.13 The far u.v. c.d. spectra of fumarase following incubation in the presence and absence of GdnHCl and subsequent dialysis

The spectra were recorded at 20 °C in 50mM sodium phosphate buffer, pH 7.3.

### 3.4 Summary of Results

The studies on pig heart fumarase suggest that the enzyme contains a considerable proportion of  $\alpha$ -helix notably, 57% as judged by circular dichroism. It was found that in the presence of low concentrations of GdnHCl the active site is extremely sensitive towards the perturbations caused by the chaotropic agent; only 45% of the activity remains in the presence of 0.1M GdnHCl. The changes in secondary and tertiary structure occur over different GdnHCl ranges; secondary structure is more stable towards the denaturant (mid-point of unfolding occurs at 1.1M GdnHCl) than tertiary structure (mid-point of unfolding occurs at 0.6M). Results from gel filtration studies indicate that in the presence of 0.5M GdnHCl the enzyme is dissociated into subunits. The picture which emerges from these data suggests that the unfolding of fumarase probably occurs in a stepwise manner in which the interactions between the subunits are disturbed at an early stage in the unfolding process leading to perturbations at the active site and dissociation into subunits followed by the loss of tertiary and secondary structure. Although the reactivation of fumarase could not be achieved by dilution of denaturant (following denaturation in concentrations of GdnHCl greater than 1M) it was found that using the dialysis procedure results in the regain of up to 62% activity relative to native enzyme providing dithiothreitol is included in the dialysis buffer. The regain of secondary structure can be achieved effectively in the presence or absence of the reducing agent using the dialysis procedure. However, the regain of tertiary structure is more efficient (approximately 94% following denaturation in 1.5M GdnHCl and subsequent dialysis) in the presence of dithiothreitol than when it is excluded from the refolding buffer (approximately 72%). Similarly the regain of quaternary structure is influenced by the presence of the reducing agent as reflected in the gel filtration data; in the absence of dithiothreitol the protein is eluted at 23ml suggesting that the enzyme is still in a monomeric state after dialysis however, when dithiothreitol is present the elution volume is the same as that of native enzyme.

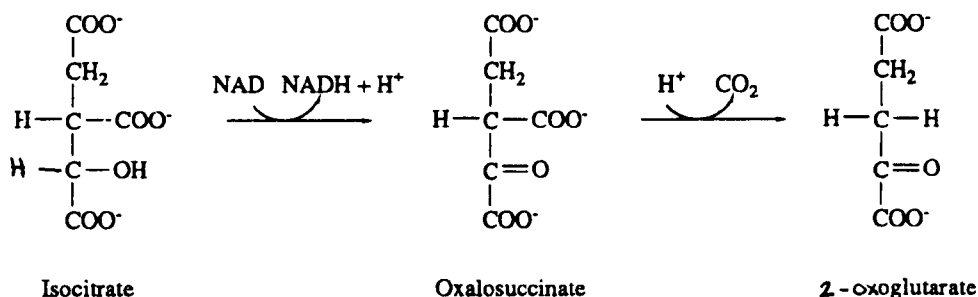


*Chapter four*

**The Unfolding and Refolding of Yeast  
Isocitrate Dehydrogenase**

## 4.1 Introduction

The oxidative carboxylation of isocitrate dehydrogenase to yield 2-oxoglutarate is catalysed by isocitrate dehydrogenases, of which there are two forms, distinguishable by their co-factor requirement for either  $\text{NAD}^+$  or  $\text{NADP}^+$ . In eukaryotes the  $\text{NAD}^+$ -specific enzyme (EC 1.1.1.41) appears to be intramitochondrial while the  $\text{NADP}^+$ -dependent enzyme (EC 1.1.1.42) is mainly extramitochondrial. The reaction catalysed by isocitrate dehydrogenase is outlined below:



The structural and kinetic properties of isocitrate dehydrogenase have been well documented. The enzyme has been isolated from various sources including *Escherichia coli*, yeast, pea mitochondria, bovine and porcine heart muscle (Hathaway and Atkinson, 1963; Giorgio *et al.*, 1970; Cox and Davies 1969; Cohen and Colman, 1971; Reeves *et al.*, 1972). The  $\text{NAD}^+$ -dependent isocitrate dehydrogenase isolated from eukaryotes has been reported to exist as an octamer, however there has been some debate about the subunit composition of the enzymes. It was proposed by Barnes *et al.* (1971) that the enzyme isolated from yeast, consisted of identical subunits of  $M_r$  approximately 39 000 as judged by SDS-polyacrylamide gel electrophoresis. Later Illingworth (1972) reported that although the enzyme showed a single band on electrophoresis in the presence of SDS, two non-identical bands, of similar  $M_r$  could be resolved in 8M urea. Keys and McAlister-Henn (1990) have recently shown using improved electrophoretic techniques, allowing greater resolution, that isocitrate

dehydrogenase is composed of two types of non-identical subunits of  $M_r$  39 000 and 40 000 respectively. Amino acid sequence analyses of the individual subunits also suggest that they contain distinct amino termini: an 11-residue sequence (Ala-Thr-Val-Lys-Gln-Pro-Ser-Ile-Gly-Gly-Tyr) of the 39 000 molecular weight subunit and a 16-residue sequence (Ala-Thr-Ala-Ala-Gln-Ala-Glu-Gly-Thr-Leu-Pro-Lys-Lys-Tyr-Gly-Gly) of the 40 000 molecular weight sub unit were obtained. Studies on  $NAD^+$ -dependent isocitrate dehydrogenase from pig heart have suggested that the enzyme contains three types of subunit of similar  $M_r$  which can be resolved by isoelectric focussing (Rutter and Denton, 1989).

The roles of the individual subunits in catalysis and regulation have been examined by a number of workers. In 1951 Kornberg, noted an unusual kinetic property of  $NAD^+$ -specific isocitrate dehydrogenase in that it would not catalyse the reverse reaction (i.e. the conversion of 2-oxoglutarate to isocitrate) under the experimental conditions employed (see Kornberg (1966). In contrast it was observed that the  $NADP^+$ -specific enzyme was able to catalyse the reaction efficiently in either direction. It was postulated that the  $NAD^+$ -enzyme could play a regulatory role in the tricarboxylic acid cycle. Further studies on the kinetics of the  $NAD^+$ -dependent enzyme from yeast revealed that an increase in the concentration of either substrate,  $Mg^{2+}$  or AMP increases the affinity of the enzyme for the others. Atkinson *et al.* (1965) suggested, using data from Hill plots of kinetic data, that the stoichiometries of binding of these effectors were equivalent to 4 molecules of isocitrate, 2 of  $NAD^+$ , 2 of divalent cation and 2 molecules of AMP *per* molecule of enzyme. These results were later confirmed by Kuehn *et al.* (1971) by direct equilibrium binding studies.  $NAD^+$ -dependent isocitrate dehydrogenase can be described as a complex oligomeric enzyme which is subject to extensive allosteric regulation (Hathaway and Atkinson, 1963).

In recent years the study of the structure and function of enzymes such as isocitrate dehydrogenase has been facilitated by the advances in recombinant DNA techniques. The isolation of the genes encoding particular enzymes and the ability to over-express

and mutate them systematically have paved the way to a better understanding of their biological significance. These techniques have not only proven to be invaluable in elucidating the role of the individual subunits of NAD<sup>+</sup>-dependent isocitrate dehydrogenase in yeast (Keys and McAllister-Henn, 1990; Cupp and McAllister-Henn, 1991, 1992, 1993) but have also served to support and extend the hypotheses of earlier workers regarding the regulatory and kinetic properties of this enzyme (Hathaway and Atkinson, 1963; Kuehn *et al.*, 1971; Illingworth, 1972).

Using immunochemical techniques and mutant strains of yeast with defects in isocitrate dehydrogenase Keys and McAllister-Henn (1990) demonstrated that the subunits of the enzyme were immunochemically distinct and that enzyme lacking in one or other of the two subunits exhibited a marked reduction in its ability to utilize isocitrate to support respiratory functions. They concluded that both types of subunits of yeast NAD<sup>+</sup>-dependent isocitrate dehydrogenase are required for the catalytic and regulatory role of this enzyme in the tricarboxylic acid cycle.

The gene encoding each type of subunit has now been cloned (Cupp and McAllister-Henn, 1991) and subsequent work using site directed mutagenesis has focused on the characterisation, function and interactions of each of the subunits (Cupp and McAllister-Henn, 1993). The amino acid sequences deduced from the genes suggest that each of the nuclear encoded subunits (IDH1 and IDH2) are synthesised as precursors of 360 and 369 amino acids, which are processed upon mitochondrial import to yield mature proteins of 349 and 354 amino acids, respectively. An allosteric model based on these genetic and kinetic studies has also been proposed to demonstrate the interactive influence of each of the subunits. This model suggests that the IDH1 subunit functions to regulate the binding of isocitrate to the IDH2 subunit and it has been postulated that this regulatory mechanism may provide a means of controlling the carbon flux through the tricarboxylic acid cycle (Cupp and McAllister-Henn, 1993).

Although many properties of NAD<sup>+</sup>-specific isocitrate dehydrogenase have been investigated, the study of the folding properties of this enzyme have not been reported. It was considered worthwhile to examine the unfolding and refolding of this enzyme in

view of the growing evidence which suggests that nuclear encoded proteins which are translocated across mitochondrial membranes and subject to further processing are difficult to refold *in vitro*, notably: pig heart fumarase (chapter 3); mitochondrial aspartate amino transferase (West and Price, 1990); citrate synthase (West *et al.*, 1990). The problems encountered in the refolding of such proteins suggests that *in vivo* other factors such as chaperones are involved in the folding process.

## **4.2 Materials and Methods**

### **4.2.1 Materials**

NAD<sup>+</sup>-dependent isocitrate dehydrogenase was purchased as a solution in 50% (v/v) glycerol from Calbiochem, Nottingham, UK.

All chemicals, substrates and reagents were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

### **4.2.2 Methods**

#### **4.2.2.1 Purification of isocitrate dehydrogenase**

The purity of the enzyme was judged by SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels. Coomassie blue staining revealed that there was a major contaminant in the commercial preparation of NAD<sup>+</sup>-dependent isocitrate dehydrogenase (accounting for approximately 35% of the sample) which migrated with the mobility expected for a protein of subunit M<sub>r</sub> of 52 000±3000. 60% of the preparation migrated with the mobility expected for isocitrate dehydrogenase, notably 38 000 ± 2000. The contaminant was removed using ion exchange chromatography on a monoQ (HR 5/5) column obtained from Pharmacia. The column was equilibrated with 40mM Tris-HCl, pH 7.6 which contained 4mM MgCl<sub>2</sub> for approximately 2h prior to use. The dialysed commercial preparation of isocitrate dehydrogenase was applied to the column and a salt gradient (0-1M NaCl) was used to separate isocitrate dehydrogenase from the contaminating material. Isocitrate dehydrogenase was eluted as a sharp single peak at a concentration of 0.17M±0.02M NaCl and the major contaminant was eluted at a concentration of 0.32±0.02M. Following chromatography the isocitrate

dehydrogenase was dialysed against 40mM Tris-HCl, pH 7.6 containing 4mM MgCl<sub>2</sub> to remove NaCl and SDS-polyacrylamide gel electrophoresis was used to assess the purity of the enzyme. At least 95% of the purified material migrated as a band of M<sub>r</sub> 38 000 ± 2000. Other workers have experienced difficulty in purifying isocitrate dehydrogenase and have reported that glycolytic enzymes can be major contaminants in the purification process.

#### **4.2.2.2 Determination of protein concentration**

The protein concentration of NAD<sup>+</sup>-dependent isocitrate dehydrogenase was determined spectrophotometrically using an absorbance value of 0.69 for a 1mg/ml protein solution at 280nm in a 1cm pathlength cell (Illingworth, 1972). The Coomassie blue binding assay (Sedmak and Grossberg, 1977) outlined in section 2.2.1, was used when the concentrations of protein were low. Control experiments showed that the protein concentrations estimated by this method were within 10% of those obtained spectrophotometrically at 280nm.

#### **4.2.2.3 Assay for NAD<sup>+</sup>-dependent isocitrate dehydrogenase**

Enzyme activity was assayed at 25 °C by monitoring the change in absorbance at 340nm corresponding to the reduction of NAD<sup>+</sup> to NADH following the conversion of DL-isocitrate to 2-oxoglutarate. Assays were run in 40mM Tris-HCl, pH 7.6 containing: 4mM MgCl<sub>2</sub>; 0.39mM DL-isocitrate; 0.33mM NAD<sup>+</sup>; 3mM dithiothreitol. The assay was initiated by addition of NAD<sup>+</sup>-dependent isocitrate dehydrogenase to 1ml of assay mix.

#### **4.2.2.4 Estimation of secondary structure of isocitrate dehydrogenase**

The data obtained from the circular dichroism spectrum of native isocitrate dehydrogenase (0.15mg/ml) were used to estimate the secondary structure content of the enzyme. The CONTIN procedure of Provencher and Glöckner (1981) was used as described in section 2.2.5 and the method of Seigel *et al.* (1980) was used for comparison (Appendix 1).

#### **4.2.2.5 Unfolding of NAD<sup>+</sup>-dependent isocitrate dehydrogenase**

The unfolding of isocitrate dehydrogenase by GdnHCl was monitored by changes in catalytic activity, circular dichroism and fluorescence.

#### **4.2.2.6 Activity in the presence of GdnHCl**

Isocitrate dehydrogenase samples (50µg/ml) were incubated in 40mM Tris-HCl pH 7.6 containing 4mM MgCl<sub>2</sub>, 0.1mM dithiothreitol and increasing concentrations GdnHCl. 20µl aliquots were removed and added to the assay mix which contained the same concentration of GdnHCl as the corresponding incubation mix.

#### **4.2.2.7 Changes in secondary and tertiary structure**

The effects of GdnHCl on the secondary and tertiary structure were monitored by circular dichroism and fluorescence respectively as outlined in section 2.2.

#### **4.2.2.8 ANS binding studies**

Enzyme samples (50µg/ml) were incubated for 15 min at 25 °C in 40mM Tris-HCl, pH 7.6, containing 4mM MgCl<sub>2</sub>, 0.1mM dithiothreitol and concentrations of GdnHCl over the range 0M to 4M. A 10µl aliquot of 2mM 8-anilino-1-naphthalenesulphonate (ANS) in buffer was added to each of the above samples. The fluorescence of each sample was monitored at 470nm (excitation at 380nm) before and after the addition of ANS. The fluorescence of free ANS in buffers containing the appropriate concentrations of GdnHCl was subtracted from each of the samples as appropriate.

#### **4.2.2.9 Refolding of NAD<sup>+</sup>-dependent isocitrate dehydrogenase**

The reactivation of enzyme was attempted, following incubation in GdnHCl, either by dilution using 40mM Tris-HCl, pH 7.6, containing 4mM MgCl<sub>2</sub> or by dialysis over a 5h period at 25 °C against two changes of this buffer. The residual concentration of GdnHCl after dilution or dialysis was judged by refractive index measurements to be less than 0.05M and 0.02M respectively. The degree of refolding of the enzyme was monitored by changes in activity, fluorescence and circular dichroism.

### **4.3 Results and Discussion**

#### **4.3.1 Secondary structure of native NAD<sup>+</sup>-dependent isocitrate dehydrogenase**

The far u.v. spectrum of isocitrate dehydrogenase is shown in Figure 4.1. The analysis of the spectrum using the CONTIN method of Provencher and Glöckner over the range 190nm to 240nm gave the following estimates of secondary structure:  $\alpha$ -helix, 36%;  $\beta$ -sheet, 29%; remainder 35%. The method of Seigel *et al.* (1980) which analyses data over the range 210nm to 240nm estimated the  $\alpha$ -helical content to be 43%. At present there is no X-ray structural information with which to compare these values although they are similar to the values obtained (from X-ray structural analyses) for other NAD<sup>+</sup>-dependent dehydrogenases (Harris and Waters, 1976).

#### **4.3.2 Unfolding of NAD<sup>+</sup>-dependent isocitrate dehydrogenase by GdnHCl**

##### **4.3.2.1 Loss of catalytic activity**

Figure 4.2 shows the loss of activity of isocitrate dehydrogenase as a function of increasing concentrations of GdnHCl. These data show that the activity of enzyme is reduced markedly at low concentrations of GdnHCl. It can be seen that the mid-point of activity loss occurred at approximately 0.15M and that no activity remained above 0.5M GdnHCl. Isocitrate dehydrogenase is therefore highly sensitive to the interactions between low concentrations of GdnHCl ( $\geq 0.1M$ ) and the active site.

##### **4.3.2.2 Changes in tertiary structure**

The effect of GdnHCl on the fluorescence of isocitrate dehydrogenase is shown in Figures 4.3. and 4.4. The former illustrates the spectra of enzyme in the presence and absence of denaturant showing that the intensity of the intrinsic fluorescence of the enzyme is markedly reduced in 6M GdnHCl and that under these conditions there is a shift in the emission maximum from 335nm to 355nm. The fluorescence at 335nm of enzyme in the presence of increasing concentrations of GdnHCl (in the range 0M to 6M) is shown in Figure 4.4. The loss of tertiary structure occurs between 0.1M and 2M GdnHCl with the mid-point of unfolding occurring at approximately 0.8M GdnHCl.



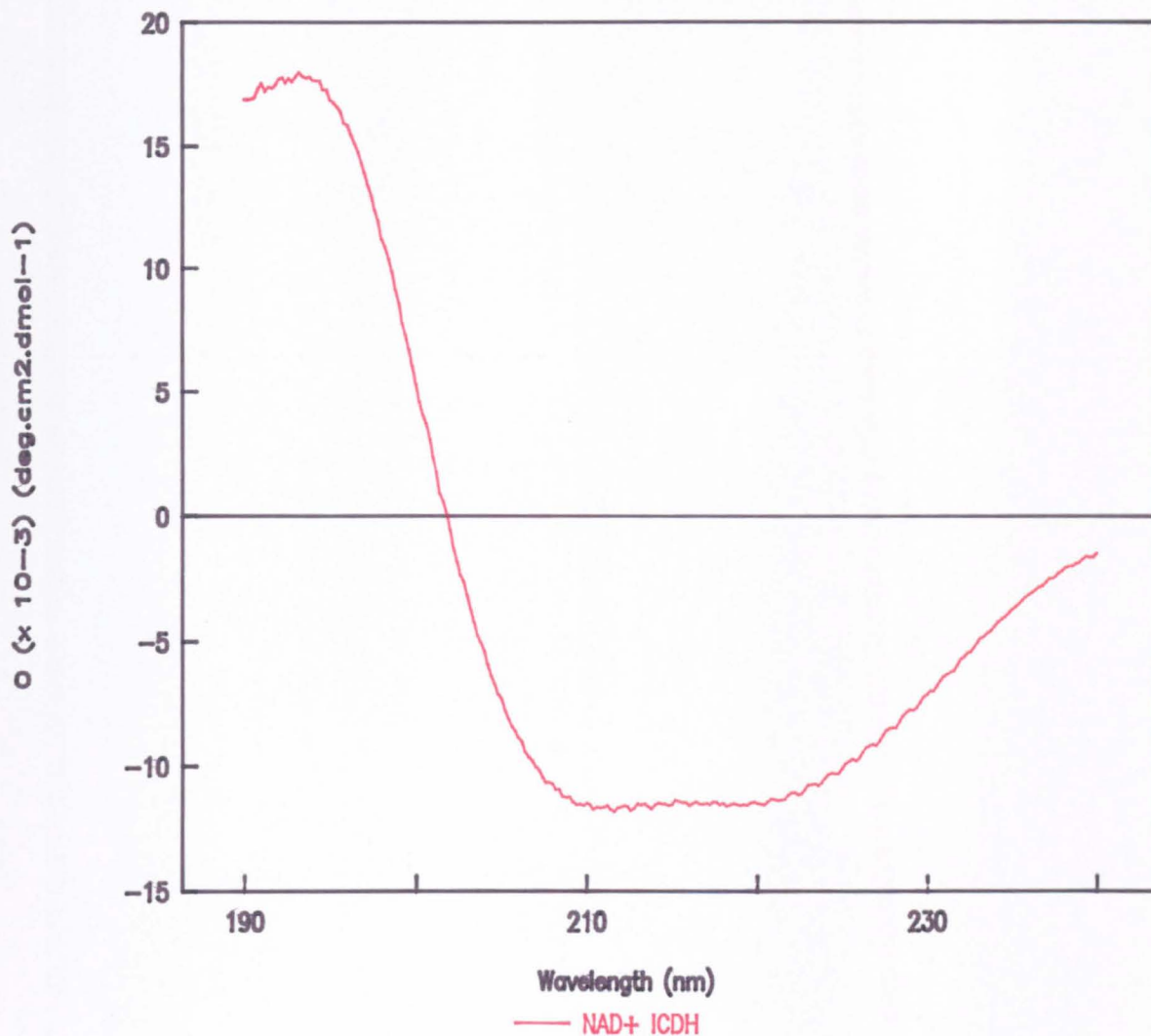
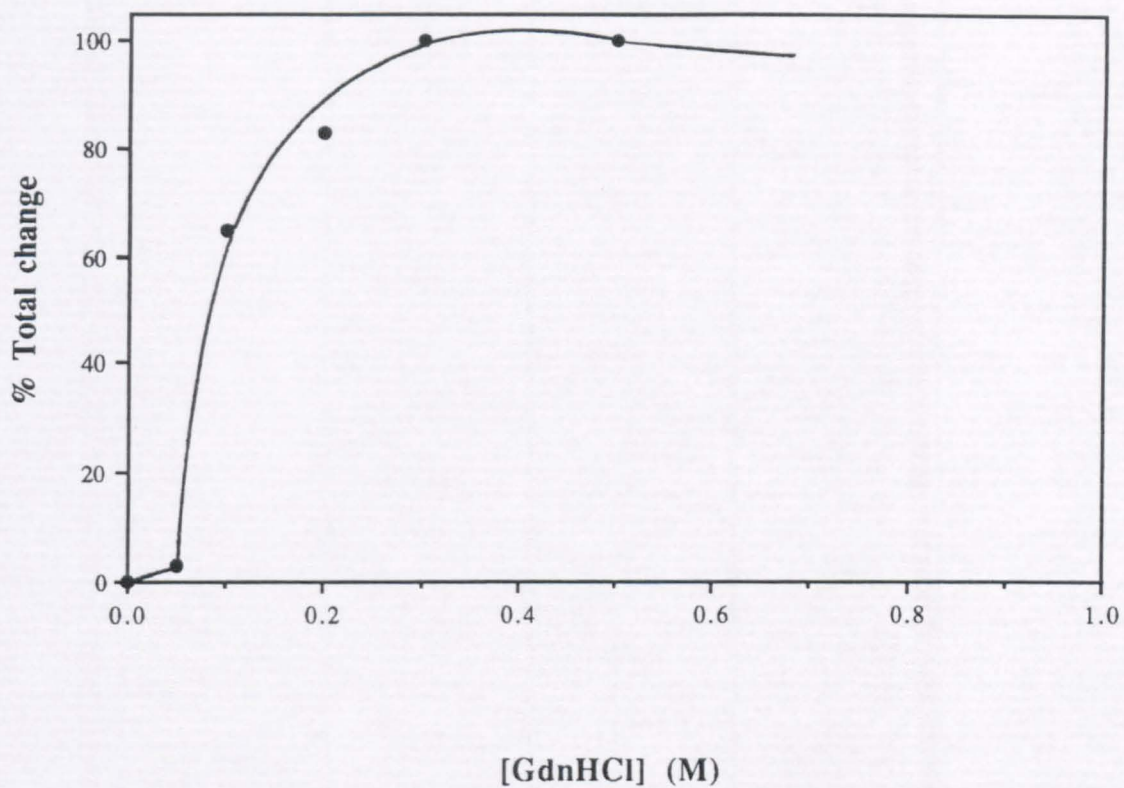


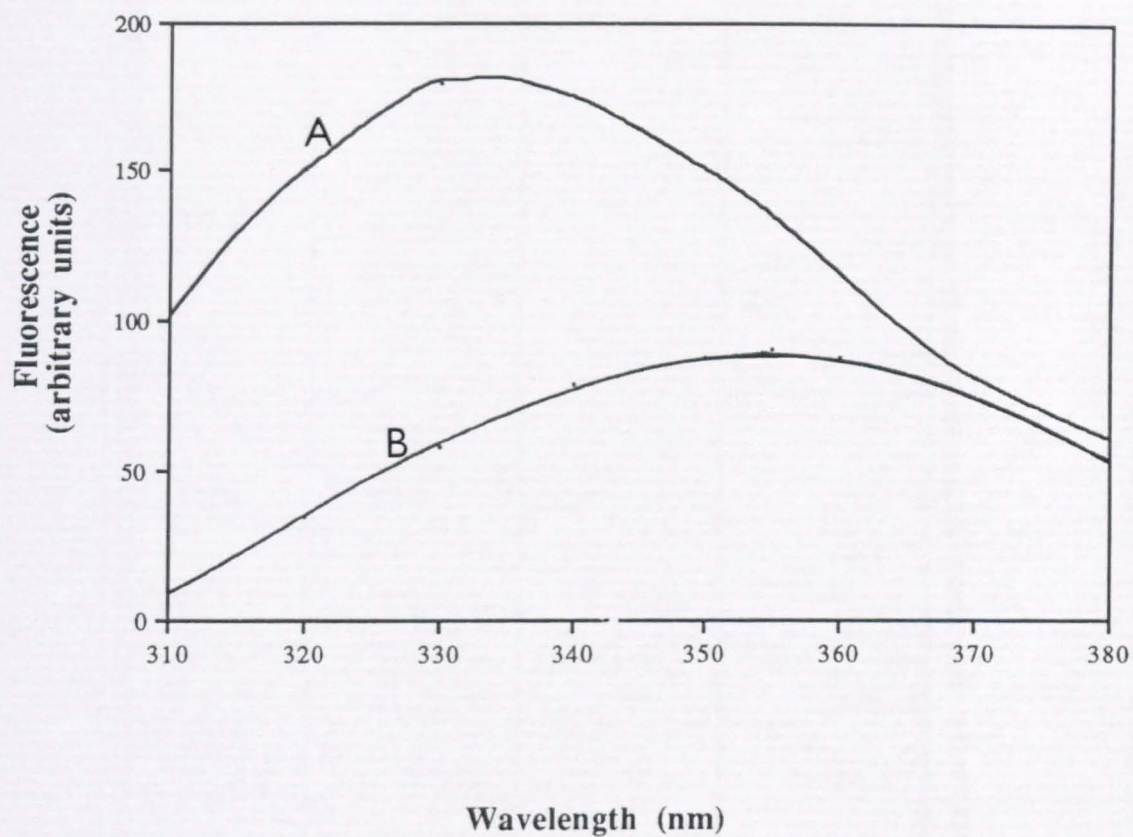
Figure 4.1 The far u.v. c.d. spectrum of NAD<sup>+</sup>-dependent isocitrate dehydrogenase

Spectra were recorded at 20 °C in 40mM Tris-HCl, pH 7.6, containing 4mM MgCl<sub>2</sub> and 0.1mM dithiothreitol.



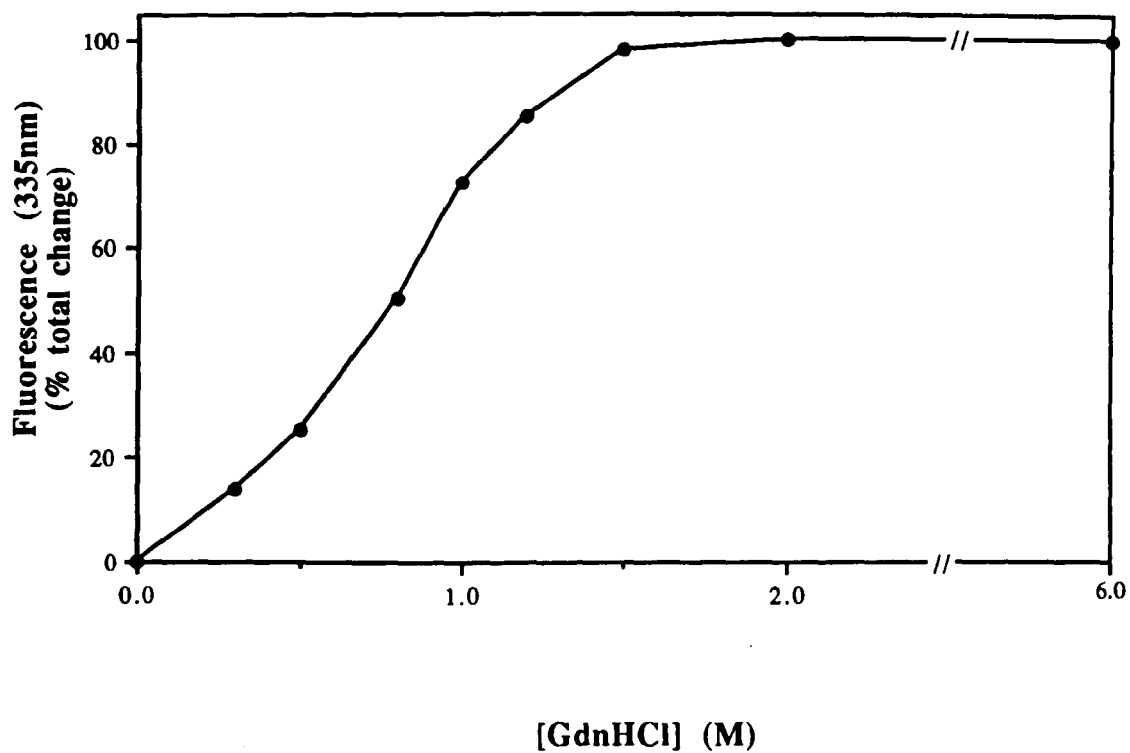
**Figure 4.2** Loss of catalytic activity of  $\text{NAD}^+$ -isocitrate dehydrogenase ( $50\mu\text{g/ml}$ ) as a function of increasing concentration of GdnHCl

Changes are expressed relative to the total change occurring between 0M and 6M GdnHCl.



**Figure 4.3** Fluorescence spectra of NAD<sup>+</sup>-isocitrate dehydrogenase (50 $\mu$ g/ml) in the absence and presence of 6M GdnHCl

The native and denatured samples (A and B respectively) were recorded at 25°C in 40mM Tris-HCl (pH 7.6), containing 4mM MgCl<sub>2</sub> and 0.1mM dithiothreitol.



**Figure 4.4** Changes in fluorescence emission at 335nm of NAD<sup>+</sup>-isocitrate dehydrogenase as a function of concentration of GdnHCl

The concentration of enzyme in each sample was 50 $\mu$ g/ml and the changes are expressed relative to the total change occurring between 0M and 6M GdnHCl.

#### 4.3.2.3 Changes in secondary structure

The circular dichroism spectra of NAD<sup>+</sup>-dependent isocitrate dehydrogenase in the presence and absence of 6M GdnHCl are shown in Figure 4.9. The enzyme in the presence of 6M denaturant is in a disordered or random state indicating that the unfolding of protein is essentially complete (Chang *et al.*, 1978). Figure 4.5. shows the changes in secondary structure as a function of increasing concentration of GdnHCl. The enzyme undergoes a considerable loss of secondary structure as the concentration of denaturant increases. The mid-point of unfolding occurs at approximately 0.9M GdnHCl and the enzyme undergoes further changes in secondary structure until 3M GdnHCl at which point the unfolding process appears to be complete.

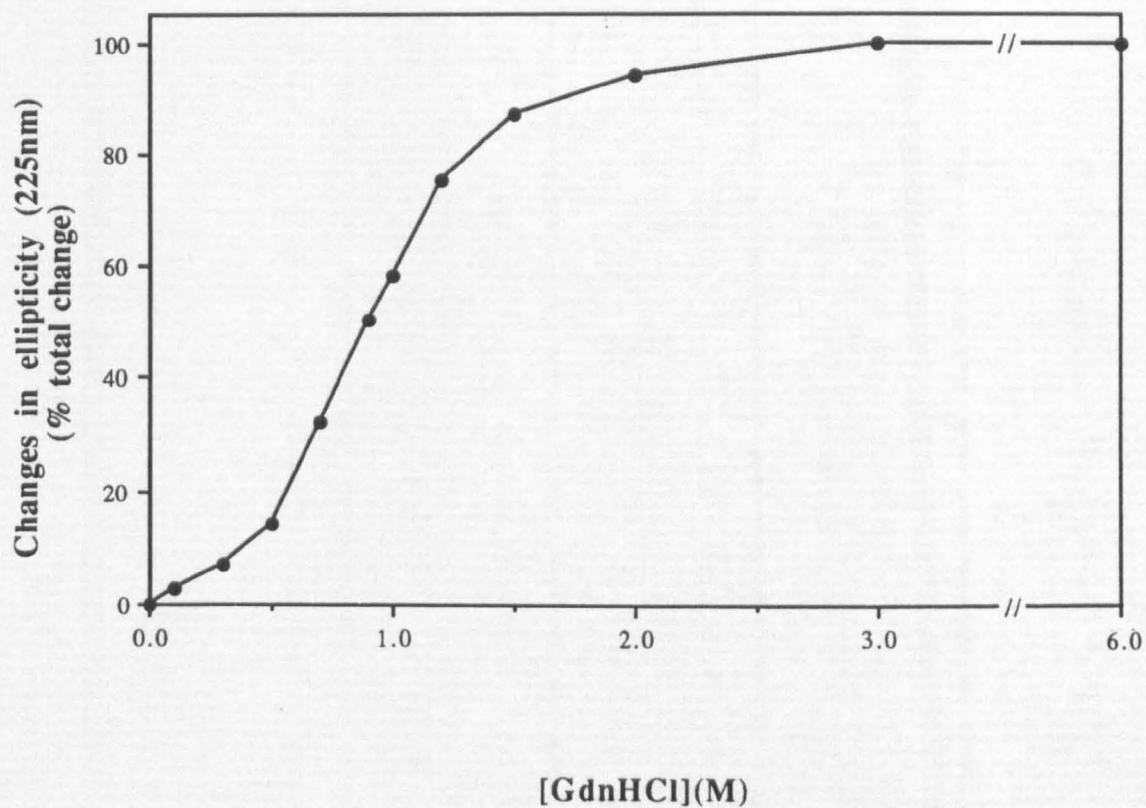
When the data relating to losses in catalytic activity, tertiary and secondary structure are combined, inactivation of isocitrate dehydrogenase occurs before any significant conformational changes are observed by fluorescence or circular dichroism (Figure 4.6).

#### 4.3.2.4 ANS binding studies

The effects of including ANS to isocitrate dehydrogenase following incubation in GdnHCl are summarised in Table 4.1:

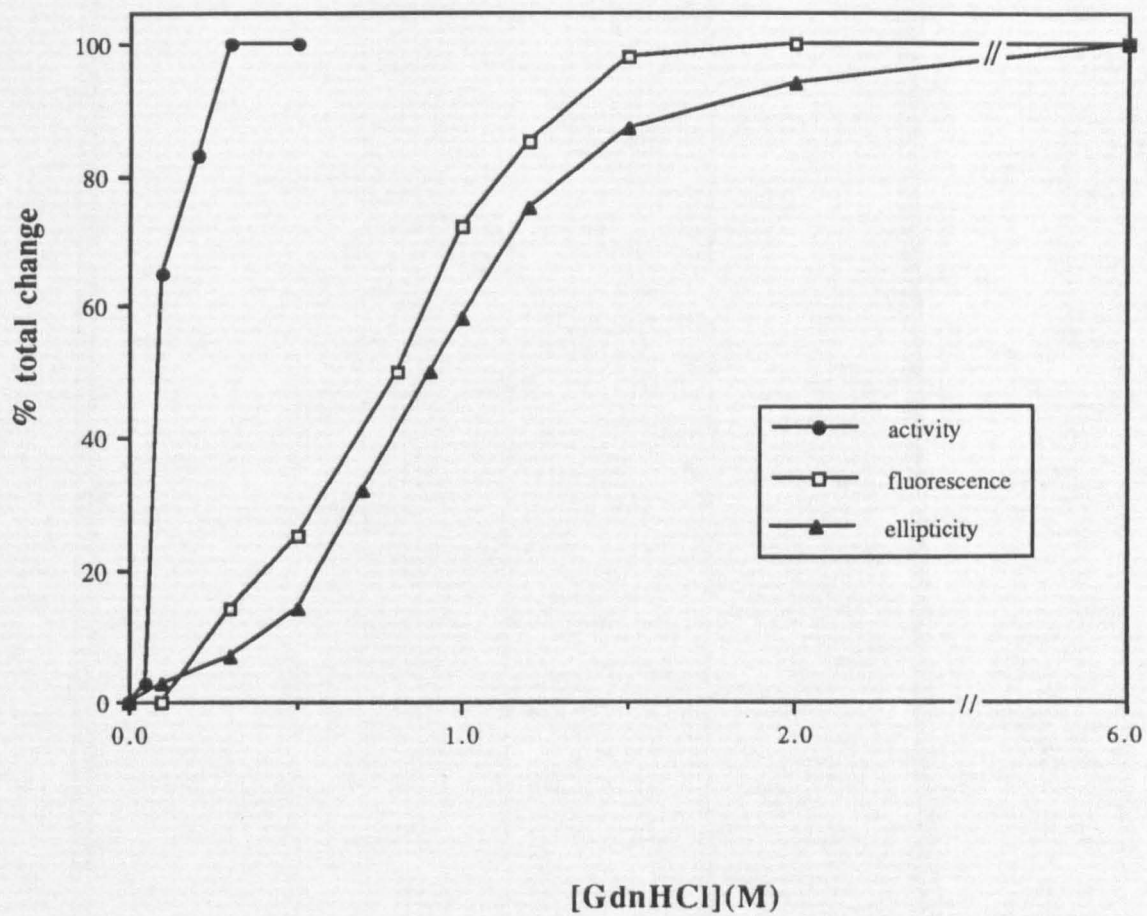
**Table 4.1 The fluorescence of ANS (470nm) following addition to NAD<sup>+</sup>-isocitrate dehydrogenase in the presence of increasing concentrations of GdnHCl**

ICDH ±[GdnHCl] (M)	ANS FLUORESCENCE (470nm) relative to free ANS (1.00)
0.0	6.770
0.5	2.881
1.0	3.699
1.5	2.029
2.0	1.927
2.5	1.489
3.0	1.461
4.0	1.689



**Figure 4.5** Changes in ellipticity at 225nm of  $\text{NAD}^+$ -isocitrate dehydrogenase as a function of increasing concentration of GdnHCl

Enzyme ( $50\mu\text{g}$ ) was incubated for 15 min at  $20^\circ\text{C}$  prior to measurement of ellipticity. Changes are expressed relative to the total change occurring between 0M and 6M GdnHCl.



**Figure 4.6** Combined data for the unfolding of  $\text{NAD}^+$ -isocitrate dehydrogenase as monitored by losses in activity, ellipticity at 225nm and fluorescence at 335nm

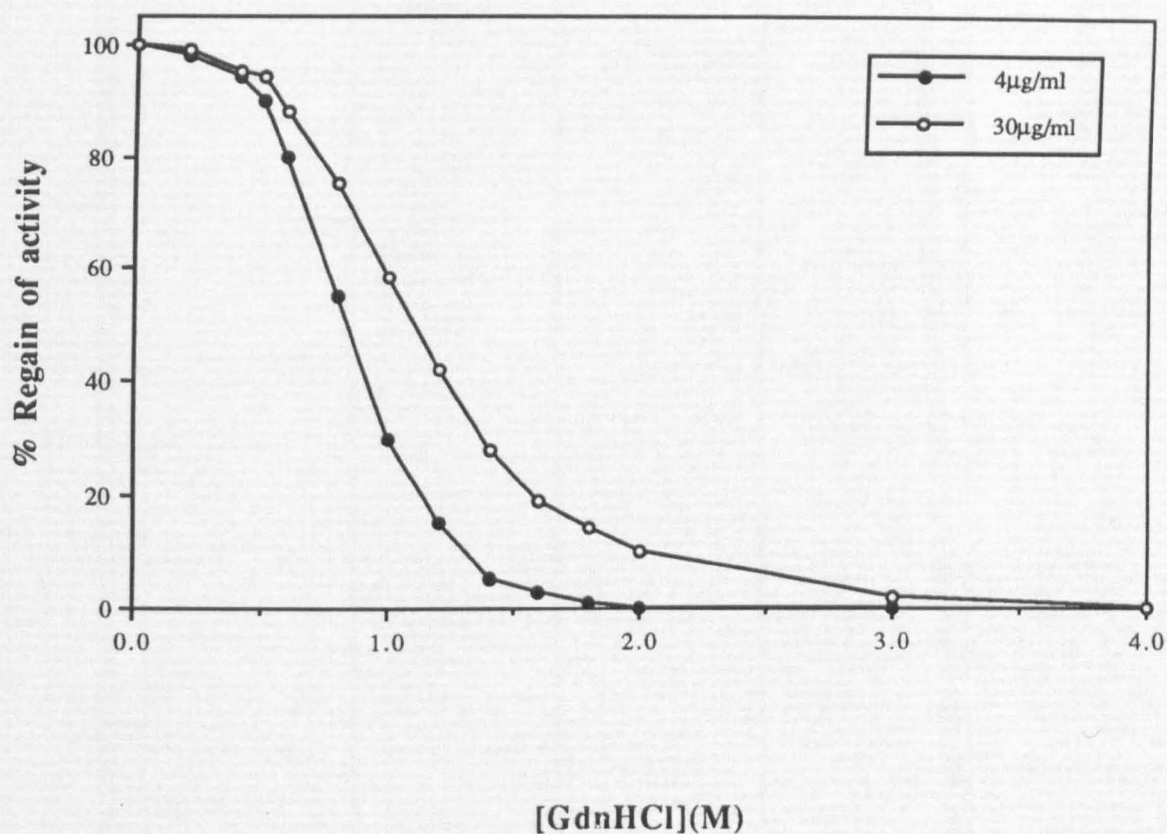
The fluorescence of ANS bound to native enzyme was found to be 6.8-fold greater than that observed with free ANS. In the presence of increasing concentrations of GdnHCl there was no enhancement in the fluorescence of the sample thus indicating that the enzyme does not adopt a significantly populated molten globule state as exhibited by other proteins under similar conditions (section 1.6.4.1; for review see Christensen and Pain 1994).

#### **4.3.3 Refolding of NAD<sup>+</sup>-isocitrate dehydrogenase by dilution**

The refolding of isocitrate dehydrogenase was attempted by dilution into/or, dialysis against, 40mM Tris-HCl containing 4mM MgCl<sub>2</sub> and 0.1mM dithiothreitol. The regain of catalytic activity following dilution (enzyme concentration, 30µg/ml) was monitored for several hours. It was found that the activity of samples, previously incubated in concentrations of GdnHCl below 2M, increased by approximately 20% between the rate measured 1min after dilution and that recorded after 60min. No further increase in activity was observed over the period between 60min and 300min. The data recorded at 120min are shown in Figure 4.7. It can be seen that the extent of regain of activity is affected by the concentration of GdnHCl in the initial incubation mix. As the concentration of denaturant was increased (over the range of 0.5M to 2M ) the ability of the enzyme to regain activity following dilution was reduced considerably. Between 2M and 4M little or no regain of activity was observed. The effect of lowering the concentration of enzyme incubated in GdnHCl was also examined. It was found that the rate of reactivation was slightly less than that observed when the concentration of enzyme was 30µg/ml. This effect has been observed for other multi-subunit enzymes and has been explained by association of monomers being favoured at higher concentrations of protein (Jaenicke, 1987).

The structural properties of the enzyme were examined, following dilution, by circular dichroism and fluorescence. The ability of the enzyme to adopt native-like secondary and tertiary structure was reduced as the concentration of GdnHCl in the initial incubation mix was increased. The data for these results have been combined and are presented in Table 4.2. It appears that beyond a critical concentration of GdnHCl ( $\leq 0.5M$ ) the refolding of isocitrate dehydrogenase is more difficult to bring about by dilution of the denaturant. At greater extents of unfolding, in the presence of increasing





**Figure 4.7** Regain of activity of  $\text{NAD}^+$ -isocitrate dehydrogenase after dilution of GdnHCl

The activities were recorded 120 min after dilution and are expressed relative to a control sample incubated in the absence of GdnHCl before dilution. The enzyme concentrations of ICDH after dilution were  $4\mu\text{g/ml}$  and  $30\mu\text{g/ml}$ .

**Table 4.2 Refolding<sup>†</sup> of NAD<sup>+</sup>-dependent isocitrate dehydrogenase after denaturation in GdnHCl and subsequent dilution**

Sample <sup>f</sup>	Activity (% native)	Fluorescence 335nm % of native	Emission Maximum (nm)	Ellipticity (225nm) % of native
Native enzyme	100	100	332	100
Enzyme + 0.5M GdnHCl	95	97	332	94
Enzyme + 1M GdnHCl	50	82	334	76
Enzyme + 3- M GdnHCl	< 2	65	337	64

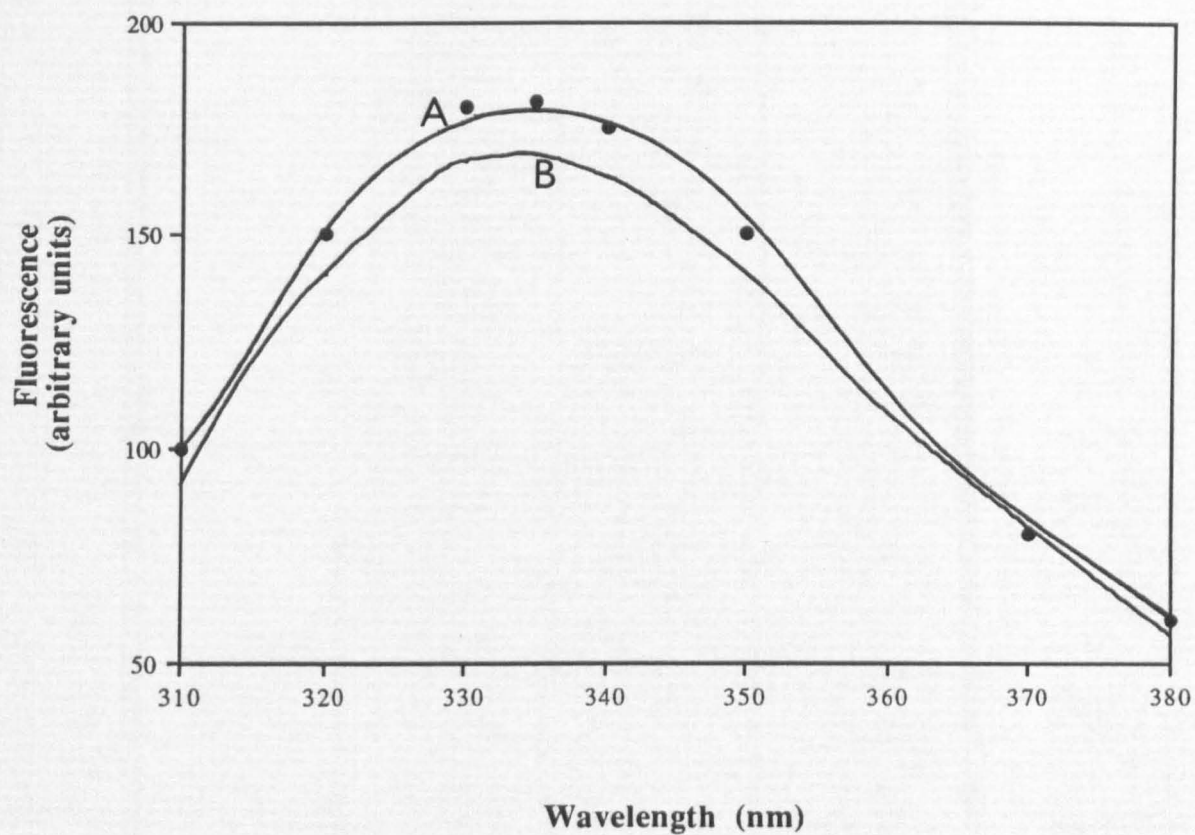
<sup>†</sup> -Refolding of the enzyme was initiated by dilution into 40 mM TrisHCl, pH 7.6, containing 4mM MgCl<sub>2</sub> and 0.1mM dithiothreitol. The enzyme concentration (after dilution) was 50µg/ml.

<sup>f</sup> - Sample descriptions refer to isocitrate dehydrogenase and the concentrations of GdnHCl at which it was incubated before dilution.

concentrations of GdnHCl, the renaturation process following dilution of the denaturant becomes less efficient. These observations have also been noted for other enzymes, for example: glutamate dehydrogenase (West and Price, 1988); fumarase (Kelly and Price, 1991). The extent of regain of structure did not increase with time beyond 60min after dilution.

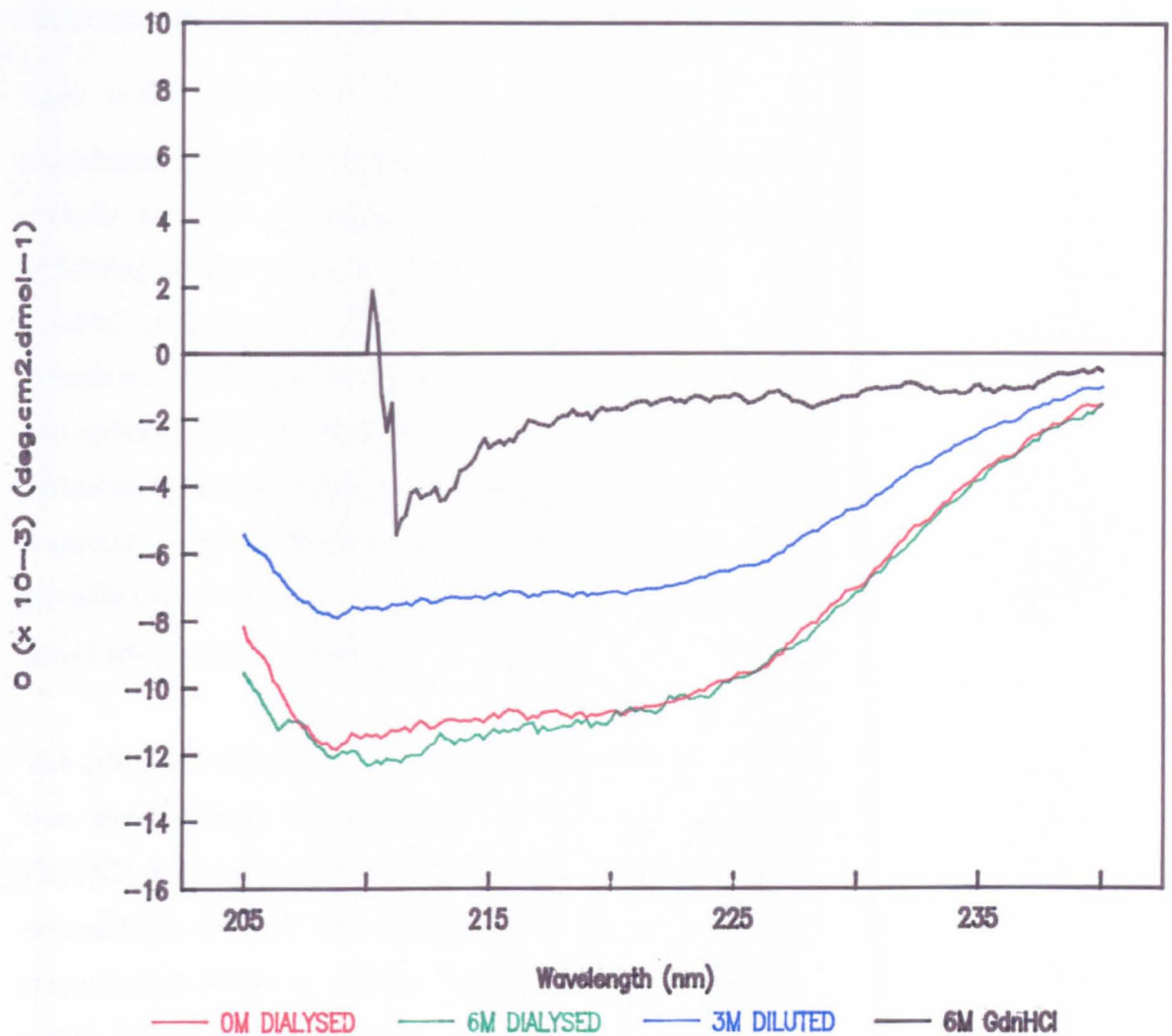
#### **4.3.4 Refolding by dialysis**

Refolding and reactivation of enzyme following incubation in GdnHCl and subsequent dialysis (to remove denaturant) was found to be more successful than that observed following the dilution procedure. Indeed, reactivation of enzyme following incubation in GdnHCl at a concentration of 6M and subsequent dialysis (enzyme concentration, 50 $\mu$ g/ml) was found to be 75% efficient. The regain of tertiary and secondary structure (Figures 4.8 and 4.9, respectively) was also found to be essentially complete following dialysis as judged by fluorescence and circular dichroism. When the concentration of enzyme was lowered (30 $\mu$ g/ml) the regain of activity was reduced to 40% although the extent of regain of structure was comparable to that observed at 50 $\mu$ g/ml.



**Figure 4.8** Fluorescence spectra of native NAD<sup>+</sup>-isocitrate dehydrogenase (A) and enzyme incubated in the presence of 6M GdnHCl and subsequently dialysed (B)

Spectra were recorded at 25°C in 40mM Tris-HCl (pH 7.6), containing 4mM MgCl<sub>2</sub> and 0.1mM dithiothreitol.



**Figure 4.9** The far u.v. c.d. spectra of NAD<sup>+</sup>-dependent isocitrate dehydrogenase following incubation in GdnHCl and subsequent dialysis or dilution

Spectra were recorded at 20 °C in 40mM Tris-HCl, pH 7.6, containing 4mM MgCl<sub>2</sub> and 0.1mM dithiothreitol. The spectra of native enzyme (0M dialysed) and the denatured enzyme (6M GdnHCl) have been included for reference. The concentration of enzyme in each sample was approximately 50µg/ml.

#### 4.4 Summary of Results

By application of the CONTIN procedure to the data obtained by far u.v. circular dichroism, NAD<sup>+</sup>-dependent isocitrate dehydrogenase has been estimated to contain 36%  $\alpha$ -helix and 29%  $\beta$ -sheet. The refolding of NAD<sup>+</sup>-dependent isocitrate dehydrogenase has been attempted using GdnHCl as denaturant followed by dilution or dialysis to lower the concentration of denaturant to less than 0.05M. During the unfolding of the enzyme major structural transitions were observed over the range of GdnHCl concentrations from 0.5M to 1.5M although loss of activity was essentially complete at concentrations  $\geq$  0.3M. The dramatic decline in catalytic activity at such low concentrations of denaturant presumably reflects the sensitivity of the active site to perturbations caused under mild denaturing conditions. This sensitivity could be due to a greater degree of flexibility at the active site of the enzyme (Tsou, 1986) although possible inhibitory effects caused by the binding of denaturant to residues situated at the active site cannot be excluded.

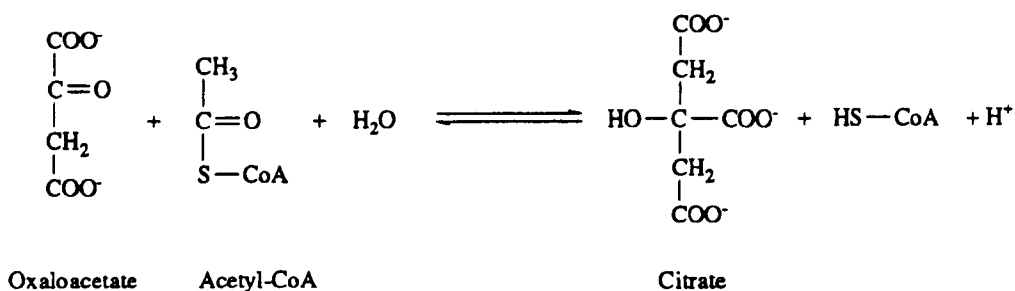
The attempted refolding of the enzyme using the dilution procedure was less successful than that obtained following dialysis. Following denaturation, in concentrations of GdnHCl greater than 0.5M, the ability of the enzyme to regain activity after dilution was substantially reduced; the regain of secondary and tertiary structure (64% and 65% respectively) following dilution indicate that the enzyme has adopted a conformation which differs significantly from that of native protein. By contrast, it was found that removal of the denaturant using the dialysis procedure yielded enzyme with a catalytic activity of up to 75% relative to the control enzyme. The secondary and tertiary structures were effectively restored following dialysis.

*Chapter five*

**The Unfolding and Refolding of Pig Heart  
Citrate Synthase**

## 5.1 Introduction

Citrate synthase (EC 4.1.3.7) occupies an important position in the tricarboxylic acid cycle where it catalyses the condensation of oxaloacetate and acetyl-CoA to form citryl CoA, which is then hydrolysed to yield citrate and CoA as shown below. This reaction effects the formation of a new carbon-carbon bond (Claisen condensation).



Citrate synthase is an example of a well characterised enzyme as a result of extensive studies on the structure, physicochemical properties and the mechanism of action of the enzyme from a variety of sources. In general two oligomeric forms of citrate synthase have been found to exist (Weitzman and Dunmore, 1969): (i) the hexameric enzyme found in gram-negative bacteria which is subject to allosteric inhibition by NADH, (Weitzman and Danson, 1976); (ii) the dimeric enzyme found in eukaryotes and gram-positive bacteria which is inhibited by ATP (Hathaway and Atkinson, 1965). The effect of ATP on the dimeric enzyme highlights the regulatory role of citrate synthase in the tricarboxylic acid cycle. The rate of the cycle is proportional to the need of the cell for ATP. When the concentration of ATP is high the  $K_m$  of citrate synthase for acetyl-CoA is increased and less citrate is formed as a result (Beeckmans, 1984).

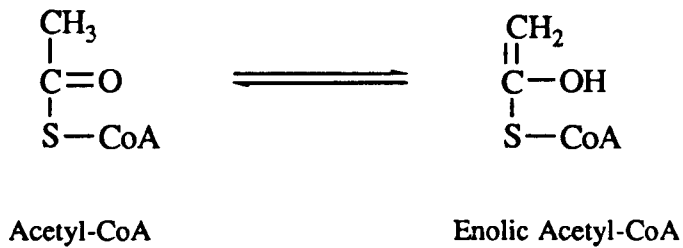
The mechanism of action of citrate synthase has been proposed on the basis of extensive structural and chemical characterisation of the enzyme. The amino acid sequence and X-ray crystallographic structure has been determined for citrate synthases from sources such as porcine and chicken heart muscle (Bloxham *et al.*, 1981; Remington *et al.*,



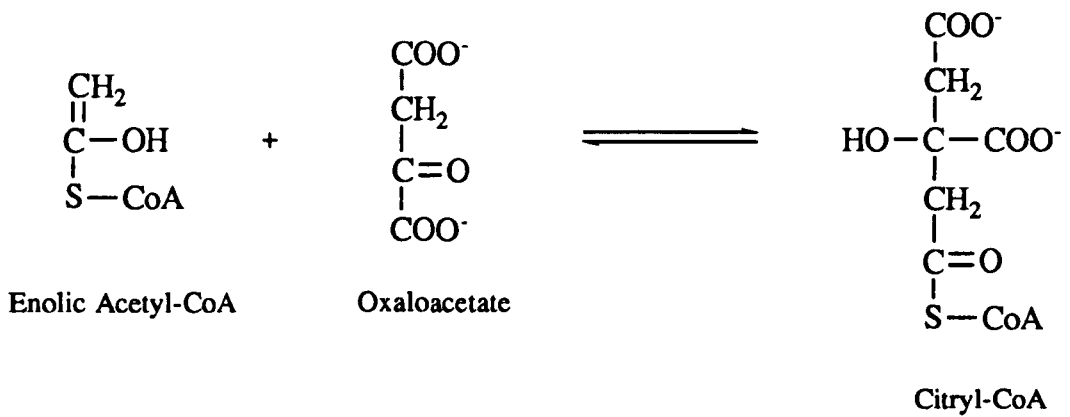
1982) and *Thermoplasma acidophilum* (Sutherland *et al.*, 1990; Russell *et al.*, 1993). Each subunit of the pig heart enzyme consists of 437 amino acid residues (Bloxham *et al.*, 1981) and together the identical subunits of approximate  $M_r$  50 000 form a tightly packed globular molecule with 40  $\alpha$ -helices per dimer. The monomers have been shown to be composed of a large and a small domain connected by a hinge region. The large and small domains contain 15 and 5 helices respectively (Remington *et al.*, 1982). These workers have also shown that the enzyme can be crystallised in two main forms, one of which is described as an “open” tetragonal and the other as a “closed” monoclinic configuration. The change in conformation between these two states has been shown to reflect the effect of the binding of substrates on the enzyme. The enzyme adopts the open configuration in the absence of substrates and the closed formation upon binding of oxaloacetate and acetyl-CoA. Indeed, crystallographic studies have shown that crystals of citrate synthase in the open conformation crack when oxaloacetate is added indicating that the binding of this substrate induces a conformational change. These findings are consistent with those of Srere (1966) who showed that the addition of oxaloacetate causes changes in the u.v. spectrum of citrate synthase. Johansson and Pettersson (1977) have shown that the binding of oxaloacetate to the enzyme increases the binding constant for acetyl-CoA by about 20-fold and it has been deduced from crystallographic studies (Remington *et al.*, 1982) that oxaloacetate binding induces a change in conformation which creates the binding site for acetyl-CoA. In turn, when both substrates have bound the small domain of each subunit rotates approximately 18 degrees relative to the large domain. This change has the effect of bringing the small domain of one subunit into contact with the large domain of the other subunit, thus positioning the enzyme in a catalytically favourable orientation, allowing each subunit to contribute functional groups to the active site of the other.

Studies using site directed mutagenesis have helped to examine the role of the amino acid side chains which are involved in catalysis at the active site of citrate synthase. His-274 and Asp-375 are two residues which are highly conserved in citrate synthases and have been implicated in the catalytic mechanism of the enzyme (Weigand and Remington, 1986). Three chemical reactions are involved in the course of citrate synthase catalysis (Figure 5.1). In the first step the methyl group of acetyl-CoA must be deprotonated to form an enol intermediate. Secondly, the enol intermediate condenses

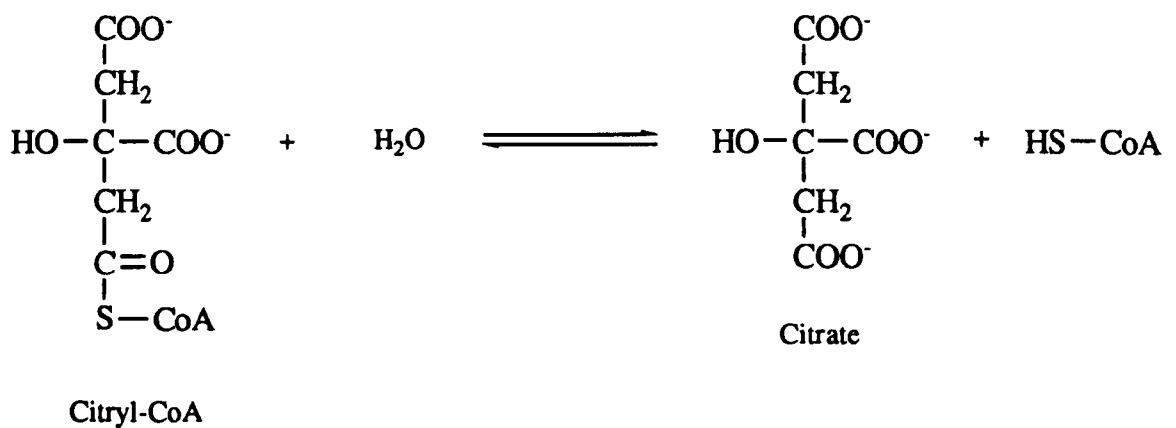
Reaction 1



Reaction 2



Reaction 3



**Figure 5.1** The three reactions catalysed by citrate synthase

with the carboxyl group of oxaloacetate to form a citryl thioester, the hydrolysis of which constitutes the third and final step of the mechanism leading to the formation of citrate and CoA. Karpusas *et al.* (1990) using X-ray crystallography data, suggested that His-274 and Asp-375 residues are involved in the enolization and condensation steps of the citrate synthase reaction. This hypothesis has since been supported by the work of Alter *et al.* (1990) who found that changing these residues by site directed mutagenesis affected the catalytic function of citrate synthase. Their studies suggested that these residues act in tandem as general acid and base catalysts respectively. In addition these workers postulated that in the condensation step His-274 accepts a proton from the hydroxyl of the enol intermediate as it condenses with oxaloacetate. Studies carried out by Zhi *et al.* (1991) have shown that mutation of His-274 to Gly causes a decrease in enzyme activity by a factor of about  $10^3$  without affecting the thermal stability of the enzyme thus suggesting that His-274 is an essential catalytic residue. Site-directed mutagenesis of the other highly conserved amino acid residues and the X-ray crystal structures of such mutants will help to elucidate the contributions of these side chains to the overall stability and mechanism of catalysis of citrate synthase.

Citrate synthase is encoded by nuclear DNA, synthesised as a precursor in the cytosol and translocated into the mitochondrial matrix. The unfolding of this enzyme has been studied by Wu and Yang (1970) and West *et al.* (1990). Preliminary studies by West *et al.* (1990) indicated that the successful renaturation of citrate synthase could not be effected by dilution of the denaturant. It was decided to investigate further the folding properties of citrate synthase following the finding that fumarase and isocitrate dehydrogenase (other translocated, mitochondrial matrix proteins) can be refolded to moderate extents when conditions are optimised in terms of protein concentration, stabilising components and method of lowering the concentration of denaturant.

## **5.2 Materials and Methods**

### **5.2.1 Materials**

Pig heart citrate synthase was purchased as an ammonium sulphate suspension from Sigma Chemical Co. and Boehringer.

GdnHCl (Ultrapure grade) was purchased from Gibco BRL, Paisley, Scotland.

Nbs<sub>2</sub> was purchased from Koch Light Co.

HEPPS (N-[2-Hydroxyethyl] piperazine-N'-[3-propane-sulphonic acid] ) and all other chemicals were obtained from Sigma.

### **5.2.2 Methods**

#### **5.2.2.1 Determination of protein concentration**

The protein concentrations of citrate synthase were determined spectrophotometrically using an absorbance value of 1.78 for a 1mg/ml protein solution at 280nm in a 1cm pathlength cell (Bloxham *et al.*, 1980). At low concentrations of protein the more sensitive Coomassie blue binding method (Sedmak and Grossberg, 1977) outlined in section 2.2.1, was used. In this method known concentrations of citrate synthase were used as a standard.

#### **5.2.2.2 Preparation of acetyl-CoA**

Acetylation of CoA was carried out as described by Stadtman (1957): 10mg of CoASH was dissolved in 1ml H<sub>2</sub>O and cooled to 0 °C; 0.2 ml of 1 M NaHCO<sub>3</sub> was then added, followed by 0.16 ml of a freshly-prepared solution of acetic anhydride (10µl acetic anhydride in 0.99 ml of H<sub>2</sub>O, cooled to 0 °C). The solution was left for 10 min at 0 °C. Acetylation was tested using Nbs<sub>2</sub>; 25µl of 10mM Nbs<sub>2</sub> was added to a solution containing 20µl acetyl-CoA + 0.98ml Tris-HCl, pH 8.0 and the absorbance at 412nm was monitored for 2 min. It should be noted that the A<sub>412</sub> of 250µM Nbs<sub>2</sub>, in buffer is approximately 0.025; this value was subtracted from the observed change in A<sub>412</sub>

#### **5.2.2.3 Assay for citrate synthase**

The activity of citrate synthase was assayed by the method of Srere *et al.* (1963). The

formation of CoA was determined by reaction with Nbs<sub>2</sub> which was measured spectrophotometrically at 412nm. The assay mixture contained 20mM Tris-HCl buffer, pH8.0, 1mM EDTA, 0.1mM oxaloacetate, 0.15mM acetyl-CoA and 0.1mM Nbs<sub>2</sub>. The reaction was initiated by adding citrate synthase to 1ml of assay mix and the rate of change of absorbance at 25 °C was recorded. The specific activity of the enzyme under these conditions was 120 μmol/min/mg.

#### **5.2.2.4 Unfolding of citrate synthase**

The unfolding of enzyme by GdnHCl was monitored by changes in catalytic activity, fluorescence and circular dichroism.

#### **5.2.2.5 Activity in the presence of GdnHCl**

Citrate synthase was incubated in 20mM Tris-HCl buffer, pH 8.0 in the absence and presence of GdnHCl at 20 °C for 15 min. Samples were taken and assayed in the same concentration of GdnHCl.

#### **5.2.2.6 Refolding of citrate synthase**

The reactivation of enzyme was attempted, following incubation in GdnHCl, either by dilution into 20mM Tris-HCl, pH 8.0 or dialysis, over a 5 hr period, against two changes of 20mM Tris-HCl pH 8.0. Studies of the effect of dithiothreitol on the refolding process were performed as described in section 3.2.2.7. The extent of refolding was examined by studies of catalytic activity, fluorescence and circular dichroism.

### **5.3 Results and Discussion**

#### **5.3.1 Secondary structure of citrate synthase**

The far u.v. circular dichroism spectrum of citrate synthase is shown in Figure 5.2. The α-helical content of the enzyme was estimated between 50 and 60% by the CONTIN procedure, (Provencher and Glöckner, 1981). This can be compared with values, obtained by other workers: 50-55% (Wu and Yang, 1970); 78% (West *et al.*, 1990); 60-80% (Zhi *et al.*, 1991). The β-sheet content was estimated to be between 40 and 50%.

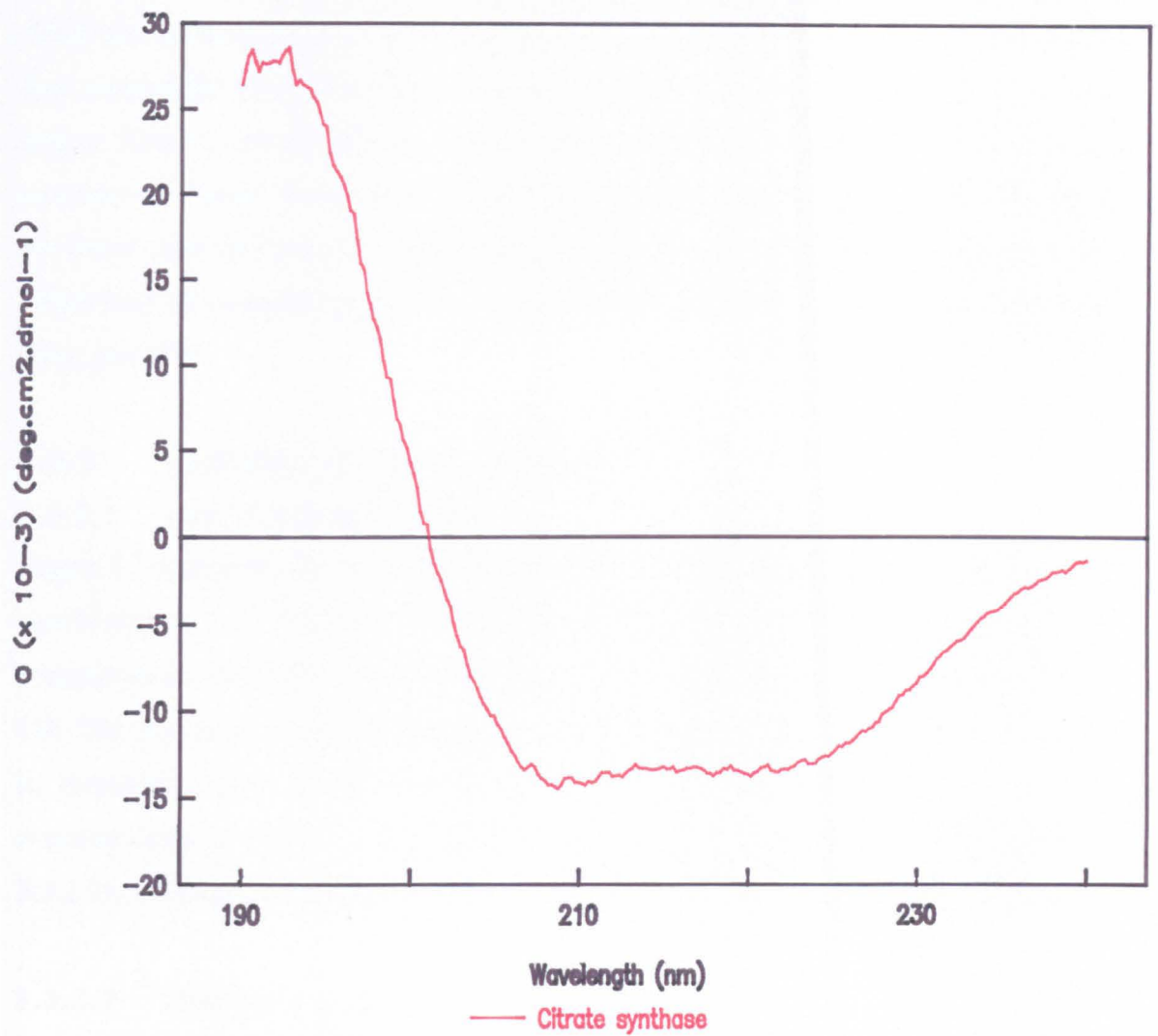


Figure 5.2 The far u.v. c.d. spectrum of citrate synthase (50 $\mu$ g/ml)

The spectrum was recorded at 20 °C in 20mM Tris-HCl buffer, pH 8.0.

The enzyme has been shown by X-ray crystallography studies to be composed of 72%  $\alpha$ -helical structure (Remington *et al.*, 1982). The helices from the two subunits are tightly packed together to give a globular structure and the dimer interface has been described by Remington *et al.* (1982) as an eight  $\alpha$ -helical “sandwich” consisting of four antiparallel pairs of helices. It has been noted by these workers that four of the helices form a smooth, slightly twisted surface which is very similar to that of the structure of  $\beta$ -sheet. It is possible that the helical values obtained by circular dichroism are lower than the X-ray crystallography data due to these unusual  $\beta$ -sheet-like helices which may be analysed incorrectly by the CONTIN procedure as representing actual  $\beta$ -sheet structure.

### **5.3.2 Unfolding of citrate synthase**

#### **5.3.2.1 Loss of catalytic activity**

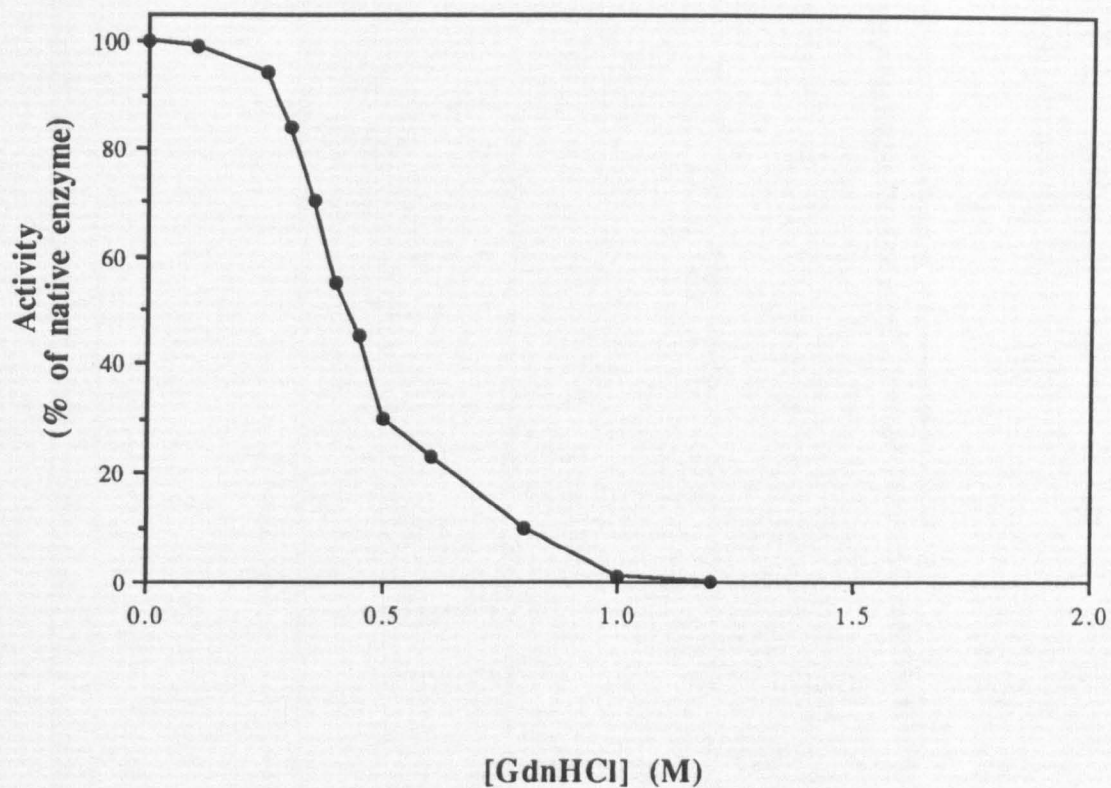
Figure 5.3 represents the loss of activity of citrate synthase in the presence of increasing concentrations of GdnHCl. The catalytic activity is reduced progressively in concentrations of GdnHCl greater than about 0.25M with little activity remaining above 1M. The mid-point of inactivation occurs at approximately 0.4M GdnHCl. The enzyme is, therefore, highly sensitive to the perturbations caused at the active site by low concentrations of GdnHCl. This sensitivity has also been noted for fumarase (section 3.3.2.1), isocitrate dehydrogenase (section 4.3.2.1) and GroEL (section 7.3.2.1).

#### **5.3.2.2 Changes in tertiary structure**

Figure 5.4 shows the unfolding of citrate synthase by GdnHCl. At lower concentrations of denaturant (between 0M and 1.5M) the intensity of fluorescence at 330nm was observed to be  $\leq 16\%$  greater than that of the native enzyme. Above 1.5M GdnHCl the unfolding of the enzyme proceeds progressively, reaching a mid-point at approximately 2.4M and an ‘end-point’ at concentrations  $\geq 4$ M GdnHCl (the intrinsic fluorescence,  $F_{330}$  of citrate synthase at the ‘end point’ is shown to be approximately 30% of the native protein and the maximum is shifted to 356nm characteristic of fully exposed tryptophan residues).

#### **5.3.2.3 Changes in secondary structure**

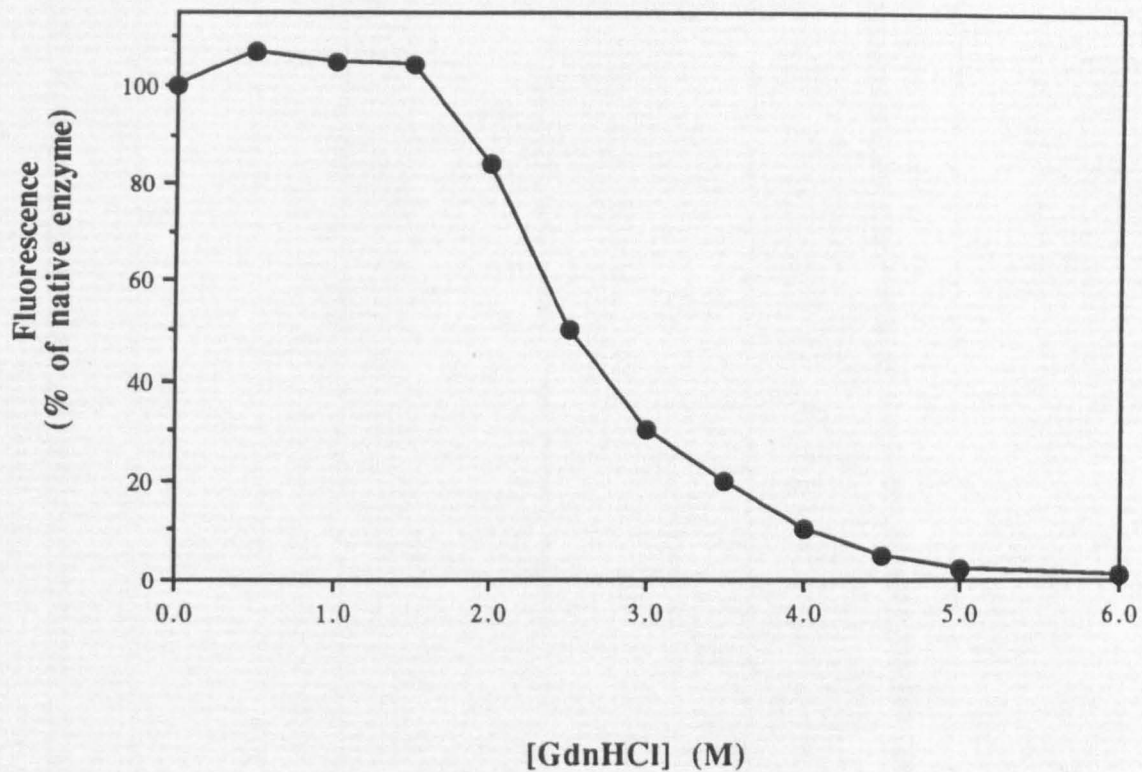
It can be seen from Figure 5.5 that the changes in secondary structure in the presence of denaturant follow a similar pattern to those observed for tertiary structure. There is an



**Figure 5.3** Loss of activity of pig heart citrate synthase in the presence of increasing concentrations of GdnHCl

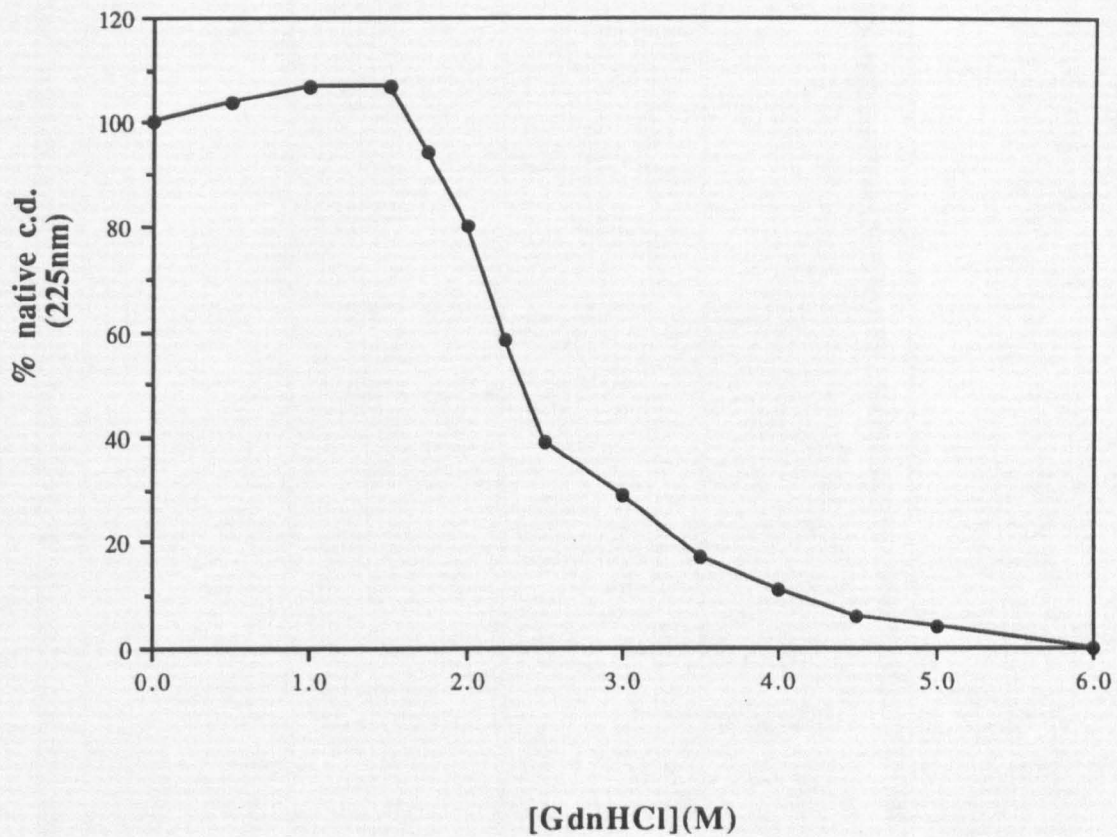
Enzyme was incubated at 20°C for 15 min in 20mM Tris-HCl (pH 8.0) containing 1mM EDTA, 0.1mM dithiothreitol and the indicated concentration of GdnHCl before a sample was taken for assay. Activities are expressed relative to native enzyme in the absence of GdnHCl.





**Figure 5.4** GdnHCl-induced changes in the tertiary structure of pig heart citrate synthase as monitored by fluorescence at 330nm

Enzyme was incubated at 20°C for 15 min in 20mM Tris-HCl (pH 8.0) containing 1mM EDTA, 0.1mM dithiothreitol and the indicated concentration of GdnHCl prior to measurement of fluorescence. The data have been “scaled” to show the relative change between 0M GdnHCl (100) and 6M GdnHCl (0).



**Figure 5.5** GdnHCl-induced changes in ellipticity at 225nm of pig heart synthase

Experimental conditions were as described in Figure 5.4. The data have been "scaled" to show the relative change between 0M GdnHCl (100) and 6M GdnHCl (0).

increase (about 10%) in the ellipticity, at 225nm, of citrate synthase in concentrations of GdnHCl up to 1.5M followed by a progressive decrease in ellipticity between 1.6M and 6M GdnHCl. The mid-point of unfolding occurs at approximately 2.35M denaturant.

It is of interest to note that the presence of GdnHCl at low concentrations has been shown to stabilise certain proteins towards thermal, urea and acid induced denaturation (Mayr and Schmidt, 1993; Hagihara *et al.*, 1993). This stabilisation is presumably afforded by the binding of guanidinium cations or chloride anions to certain amino acid side chains, thus shielding them from the effects of low pH, urea and increases in temperature. Furthermore, Hagihara *et al.* (1993) have shown that concentrations of GdnHCl up to 1M are able to effect the refolding of acid-induced unfolded apomyoglobin and cytochrome c by stabilising the molten globule state. Their c.d. data suggest that the molten globule state of apomyoglobin exhibits an increase (approximately 20%) in secondary structure relative to the native protein. It is possible that in the case of citrate synthase the apparent increases in secondary structure and tertiary structure reflect the interactions of low concentrations of GdnHCl with amino acid side chains which by altering the solute/solvent interactions may allow additional structural elements to form.

### **5.3.3 Reactivation of citrate synthase**

#### **5.3.3.1 Reactivation by dilution of denaturant**

Following denaturation in 6M GdnHCl for 15 min and subsequent dilution into 20mM Tris-HCl, pH 8.0, containing 1mM EDTA the activity of citrate synthase (4 $\mu$ g/ml, following dilution) was monitored over a period of 24 hr. It was found that no activity could be regained under these conditions, a result which is in accord with that of West *et al.* (1990). By extending the studies of West *et al.* (1990) it was found that the ability of citrate synthase to refold, following dilution of denaturant, was influenced by factors such as protein concentration, the presence of dithiothreitol and EDTA in the buffer and the inclusion of BSA in the reactivation mix. Table 5.1 and Figure 5.6 summarise the reactivation data obtained by varying the renaturation conditions and show that a maximum reactivation of approximately 55% was obtained when the final enzyme concentration (following dilution) was approximately 5 $\mu$ g/ml and the buffer was 50mM Hepps, pH 8.0 containing, 1mM EDTA, 0.1mM dithiothreitol and BSA (1mg/ml). The

**Table 5.1** Conditions affecting the regain of activity of pig heart citrate synthase following dilution or dialysis of denaturant

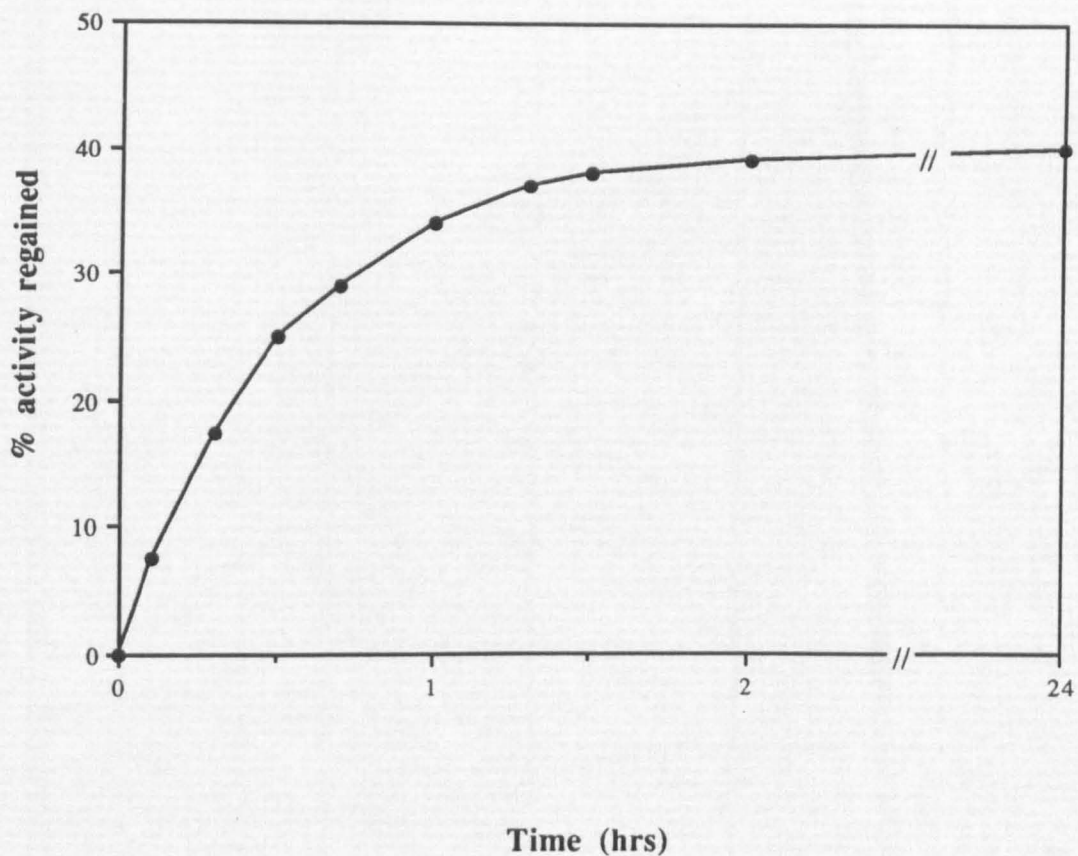
Refolding conditions	Regain of $\approx$ activity (%)
<u>By dilution into:</u> <sup>f</sup>	
20mM TrisHCl 1mM EDTA (pH 8.0)	< 1
20mM TrisHCl 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol	42
20mM TrisHCl 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol + BSA (1mg/ml)	46
50mM Hepps 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol	52
50mM Hepps 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol + BSA (1mg/ml)	55
<u>By dialysis into:</u> <sup>†</sup>	
20mM TrisHCl 1mM EDTA (pH 8.0)	< 1
20mM TrisHCl 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol	59
50mM Hepps 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol	44

*notes:*

$\approx$  - All experiments were carried out in triplicate and the activity data shown represent the mean values obtained

*f* - Concentration of enzyme following dilution was approximately 5 $\mu$ g/ml

$\dagger$  - Concentration of enzyme following dialysis was approximately 10 $\mu$ g/ml



**Figure 5.6** Reactivation of pig heart citrate synthase following GdnHCl-induced denaturation and subsequent dilution

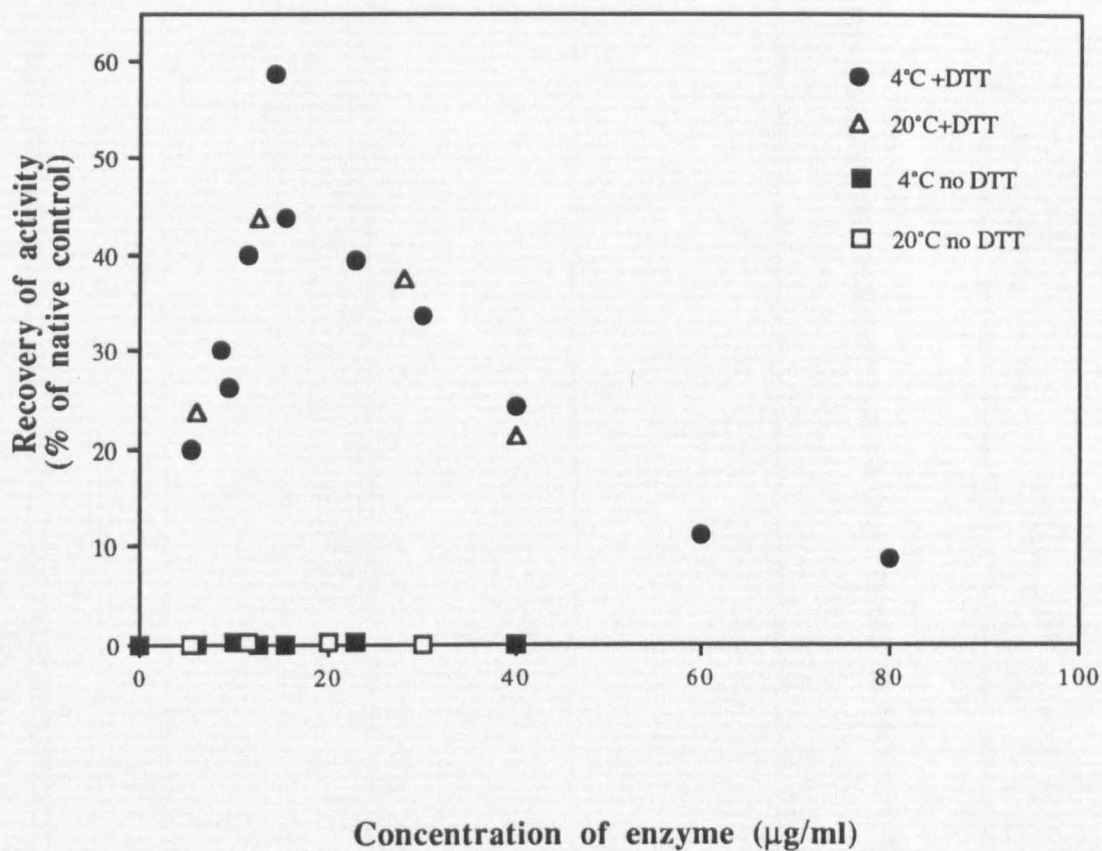
Enzyme was incubated in 6M GdnHCl for 15 min prior to dilution with 50mM Hepps-Na<sup>+</sup>/1mM EDTA buffer (pH 8.0). Samples were assayed for citrate synthase activity for up to 24hr after dilution.

low concentration of protein and the strong absorbance of Hepps in the far u.v. precluded the examination of the structural properties of the enzyme following dilution.

### 5.3.3.2 Reactivation by dialysis of denaturant

Table 5.1 and Figure 5.7 show the extent of reactivation of citrate synthase following denaturation in 6M GdnHCl and subsequent dialysis (section 5.2.2.6). The data in Figure 5.7 are presented as a function of enzyme concentration and show that reactivation is optimal (about 55-60% of native enzyme) when the initial concentration of enzyme before dialysis is in the range from about 10 to 20 $\mu$ g/ml and dithiothreitol is present in the dialysis buffer. In the absence of the reducing agent less than 1% reactivation is achieved following dialysis reflecting the importance of maintaining the integrity of the cysteine side chains of citrate synthase during the unfolding and refolding of the enzyme. At concentrations of enzyme less than 10 $\mu$ g/ml and greater than 25 $\mu$ g/ml there is a marked reduction in the extent of regain of activity. It has been noted that citrate synthase exhibits a tendency to dissociate reversibly into monomers at low protein concentrations (McEvily and Harrison, 1986) which could account for the reduction in the extent of regain of activity in these studies. The decline in reactivation at higher concentrations, together with the observation that the enzyme solution appears cloudy following dialysis, suggests that aggregation is a competing process under these conditions (Jaenicke, 1987, 1993). The tendency of citrate synthase to form aggregates following the dilution of denaturant has also been noted by Buchner *et al.* (1991). Measurements of the concentration of protein following dialysis indicated that below about 15 $\mu$ g/ml the recovery of soluble protein was in the range from 90 to 100% whereas with increasing concentrations of protein there was a steady decline in the percentage recovery of protein (60% at 30 $\mu$ g/ml and approximately 30% at 80 $\mu$ g/ml). These data provide further evidence for the formation of aggregates at the higher concentrations of protein.

There is little or no apparent effect of changing the temperature at which dialysis is performed on the extent of reactivation. This observation contrasts with the results obtained for some other proteins under similar conditions. For example, the extent of refolding of the chaperonin GroEL (chapter 7) is reduced by at least 30% following dialysis at 2 °C compared with dialysis at 20 °C. Conversely, the efficiency of refolding



**Figure 5.7 Recovery of activity of citrate synthase following denaturation in 6M GdnHCl and subsequent dialysis**

The concentration of enzyme indicated corresponds to that before dialysis. The recovery of activity was calculated relative to a control sample containing no GdnHCl and corrected for the increase in volume which occurs on dialysis.

of rhodanese (Gatenby *et al.* 1990) and dimeric ribulose 1,5-bisphosphate carboxylase (Viitanen *et al.* 1990) decreases markedly as the temperature is raised.

### 5.3.3.3 Regain of secondary structure

Figure 5.8 shows the extent of refolding of citrate synthase, in terms of secondary structure, following dialysis of denatured enzyme. At an enzyme concentration of 10 $\mu$ g/ml the regain of native secondary structure is almost 100% complete when dithiothreitol is present. The extent of regain of secondary structure is markedly reduced when the reducing agent is omitted from the dialysis buffer. It is, therefore, not surprising that reactivation of the enzyme does not occur under these conditions. At a higher enzyme concentration of 37 $\mu$ g/ml the shape of the c.d. spectrum following dialysis is strikingly different from that of the native protein. A single minimum at 225nm is observed at the higher concentration and the intensity of signal is about 20% that of the native enzyme. It is highly probable that this behaviour is due to the formation of aggregates; the scattering of the incident radiation would lead to distortions of the c.d. spectra of proteins (Adler *et al.*, 1973).



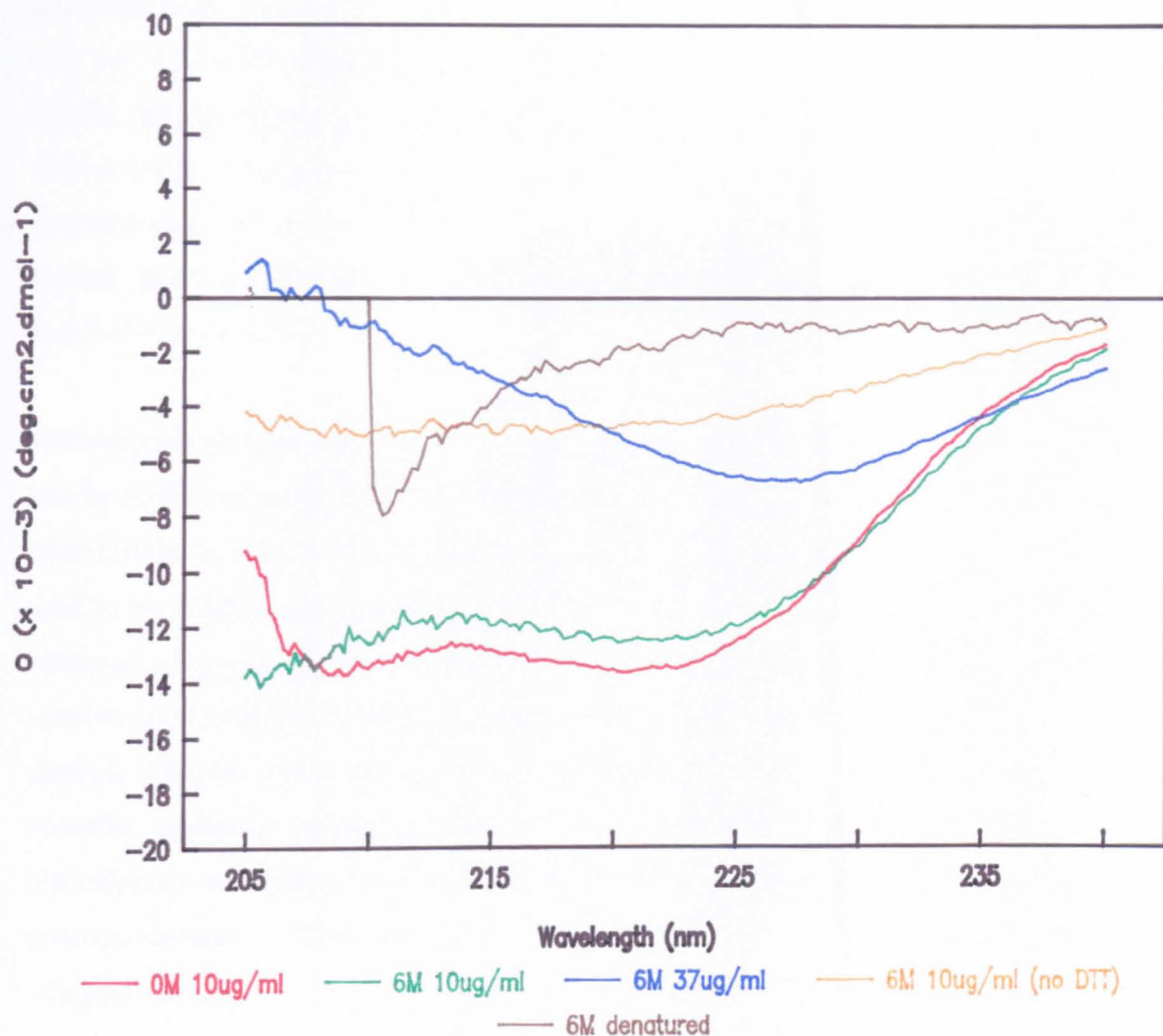


Figure 5.8 The far u.v. c.d. spectra of citrate synthase following incubation in absence or presence of 6M GdnHCl and subsequent dialysis

The spectra were recorded at 20 °C in 20mM Tris-HCl buffer, pH 8.0 (at the indicated protein concentrations). The spectrum of denatured citrate synthase has been included for reference.

## 5.4 Summary of Results

Porcine heart citrate synthase was estimated to contain between 50 and 60%  $\alpha$ -helix following analyses of data obtained by far u.v. circular dichroism. The active site of the enzyme is sensitive towards the presence of GdnHCl at concentrations greater than 0.25M. Interestingly, an increase in both the ellipticity, at 225nm, and fluorescence, at 330nm (of approximately 10 and 16% respectively) occurs in the presence of GdnHCl concentrations of up to 1.5M. Above this concentration the loss of secondary and tertiary structure run broadly in parallel; the mid-points of unfolding occurring at 2.35 and 2.40M respectively.

Following dilution of denaturant a considerable amount of catalytic activity (up to 55%) can be recovered using optimised conditions. The extent of reactivation is affected by such factors as enzyme concentration, the presence of a reducing agent and the buffer used in the dilution step. Approximately 55-60% reactivation of citrate synthase can be achieved using the dialysis procedure; the extent of reactivation is similarly dependent upon the concentration of enzyme, the presence of dithiothreitol and the buffer used for dialysis. Circular dichroism spectra revealed that the regain of secondary structure is virtually complete following dialysis using optimised refolding conditions. If dithiothreitol is omitted from the dialysis buffer the regain of secondary structure is reduced markedly. When the concentration of enzyme is raised to approximately 37 $\mu$ g/ml there is a dramatic reduction in the regain of secondary structure; the c.d. spectrum varies considerably from native enzyme and there is a 80% reduction in the ellipticity value obtained at 225nm.

*Chapter six*

**The Unfolding and Refolding of Citrate Synthase  
from *Thermoplasma acidophilum* and  
*Acinetobacter anitratum***

## 6.1 Introduction

Comparative studies of the citrate synthases from eukaryotes, eubacteria and archaeobacteria have highlighted their diversity in terms of amino acid sequence, subunit structure and catalytic activity (Weitzman and Danson, 1976; Henneke *et. al.*, 1989). As mentioned in the introduction to chapter 5 eukaryotes and Gram-positive bacterial citrate synthases are dimeric and show isosteric regulation in response to ATP whereas the Gram-negative bacterial citrate synthases are hexameric and show allosteric regulation in response to NADH. The subunits from both types of enzymes have been shown to be similar with the hexameric enzyme functioning as a trimer of the basic dimer (Else *et. al.*, 1988).

In view of the structural diversity between citrate synthases from eukaryotes, eubacteria and archaeobacteria it was considered worthwhile to examine the structural properties and relative stabilities of these enzymes, isolated from a source from each of these groups, towards thermal and chemical denaturation. The enzyme from pig heart was examined in chapter 5 and the results obtained will be discussed in comparison with data presented in this chapter. Citrate synthase from *Acinetobacter anitratum* is a hexameric enzyme and although there is no detailed information on the structure of this eubacterial enzyme it has been shown to possess 70% and 23% sequence identity with the enzymes from *E. coli* and pig heart respectively. Recent findings have suggested that *E. coli* (and possibly other Gram-negative bacteria) may possess a second citrate synthase with the N-terminal amino acid sequence showing significant identity with the enzymes from other archae bacteria *Thermoplasma acidophilum* is a thermophilic archaeobacterium which has an optimal growth temperature of 55 °C. The citrate synthase from this organism is similar to the pig heart enzyme in that it is a dimer, however the subunit is smaller ( $M_r$  43 000) than that of pig heart. Sequence studies have shown that the N-terminal 46 residues of the pig heart enzyme are absent from the *Tp. acidophilum* enzyme. These studies have also shown the enzyme to possess approximately 28% and 20% identity with citrate synthases from *Ac. anitratum* and pig heart respectively.

Despite this low sequence identity 8 of the 11 residues involved in the catalytic mechanism of pig heart citrate synthase are conserved in *Tp. acidophilum* and two others are involved in conservative substitutions (Sutherland *et al.*, 1990). Crystals from the *Tp. acidophilum* enzyme have been obtained and their structural characterisation is currently in progress (Russell *et. al.*, 1993).

## **6.2 Materials and Methods**

### **6.2.1 Materials**

Citrate synthases from *Ac. anitratum* and *Tp acidophilum* were kindly provided by Dr David W. Hough (School of Biology and Biochemistry, University of Bath, Bath, England) with whom a collaborative study on the stabilities of citrate synthases was established. The purification of both enzymes involved expression of the cloned genes in *E. coli* followed by dye ligand chromatography.

Hepps buffer and succinimide were obtained from Sigma chemical company. Succinimide was recrystallised from ethanol prior to use.

### **6.2.2 Methods**

#### **6.2.2.1 Determination of protein concentration**

The protein concentrations of citrate synthases from *Tp. acidophilum* and *Ac. anitratum* were determined from absorbance measurements at 280nm using  $A_{280}$  (1mg/ml) coefficients of 1.126 and 0.941 respectively. These values were calculated on the basis of the number of tryptophan and tyrosine residues for each enzyme. At the lower concentrations of protein the method of Sedmak and Grossberg (1977) was used as outlined previously (section 2.2.1).

#### **6.2.2.2 Assay for citrate synthase**

Citrate synthases from *Ac. anitratum* and *Tp. acidophilum* were assayed as described in section 5.2.2.3 except that the assay temperature in the case of the latter was 55 °C.

#### **6.2.2.3 Thermal stabilities of citrate synthase**

The thermal stabilities of the enzymes from pig heart *Ac. anitratum* and *Tp. acidophilum* were measured by monitoring the changes in ellipticity at 225nm as a function of increasing temperature. Solutions were allowed to equilibrate for approximately 4 min before their mean ellipticity values (at 225nm) were calculated from a minimum of 10 readings. A microprobe connected to a Jenway 2003 digital monitor was used to measure the temperature of solutions inside the cuvette.

#### **6.2.2.4 Differential scanning calorimetry**

Differential scanning calorimetry (d.s.c.) experiments were kindly performed by Dr Alan Cooper (Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, Scotland) over the range 20 °C -95 °C using a Microcal MC-2D instrument at a scan rate of 60 °C/hr. Protein solutions (0.9-1.3mg/ml) in 50mM Hepps/Na<sup>+</sup>, pH 8.0, containing 1mM EDTA, and reference buffers were degassed briefly prior to loading.

#### **6.2.2.5 Quenching of tryptophan fluorescence by succinimide**

Quenching of citrate synthase fluorescence by succinimide was performed using the method described by Eftink and Ghiron (1984). Aliquots of a stock solution (2.5M) of succinimide were added to 1ml of citrate synthase in 50mM Hepps-Na<sup>+</sup>/ 1mM EDTA (pH 8.0) in the absence or presence of 6M GdnHCl. On addition of each aliquot of quencher the fluorescence emission at 330nm (excitation 290nm) was recorded and each value was corrected for the dilution factor. The results were expressed as Stern-Volmer plots in which the concentration of succinimide is plotted against the ratio of the initial fluorescence and the fluorescence following the addition of quencher ( $F_0 / F$ ). The initial slopes of these plots give the Stern-Volmer constant for succinimide quenching ( $K_S$ ).

It should be noted that all experiments described in this chapter were carried out in both 20mM Tris-HCl /1mM EDTA (pH 8.0) and 50mM Hepps-Na<sup>+</sup>/1mM EDTA (pH 8.0). Unless otherwise stated the results in each buffer system were broadly similar.

### **6.3 Results and Discussion**

#### **6.3.1 Secondary structure of *Ac. anitratum* and *Tp. acidophilum* citrate synthases**

The far u.v. spectra were recorded in 20mM Tris HCl, pH 8.0 and are shown in Figure 6.1. The spectrum obtained for the pig heart enzyme (section 5.3.1) has been superimposed for comparison. The results obtained suggest that the citrate synthases from both pig heart and *Tp. acidophilum* have similar secondary structure conformations with the c.d. spectra being essentially superimposable. The CONTIN

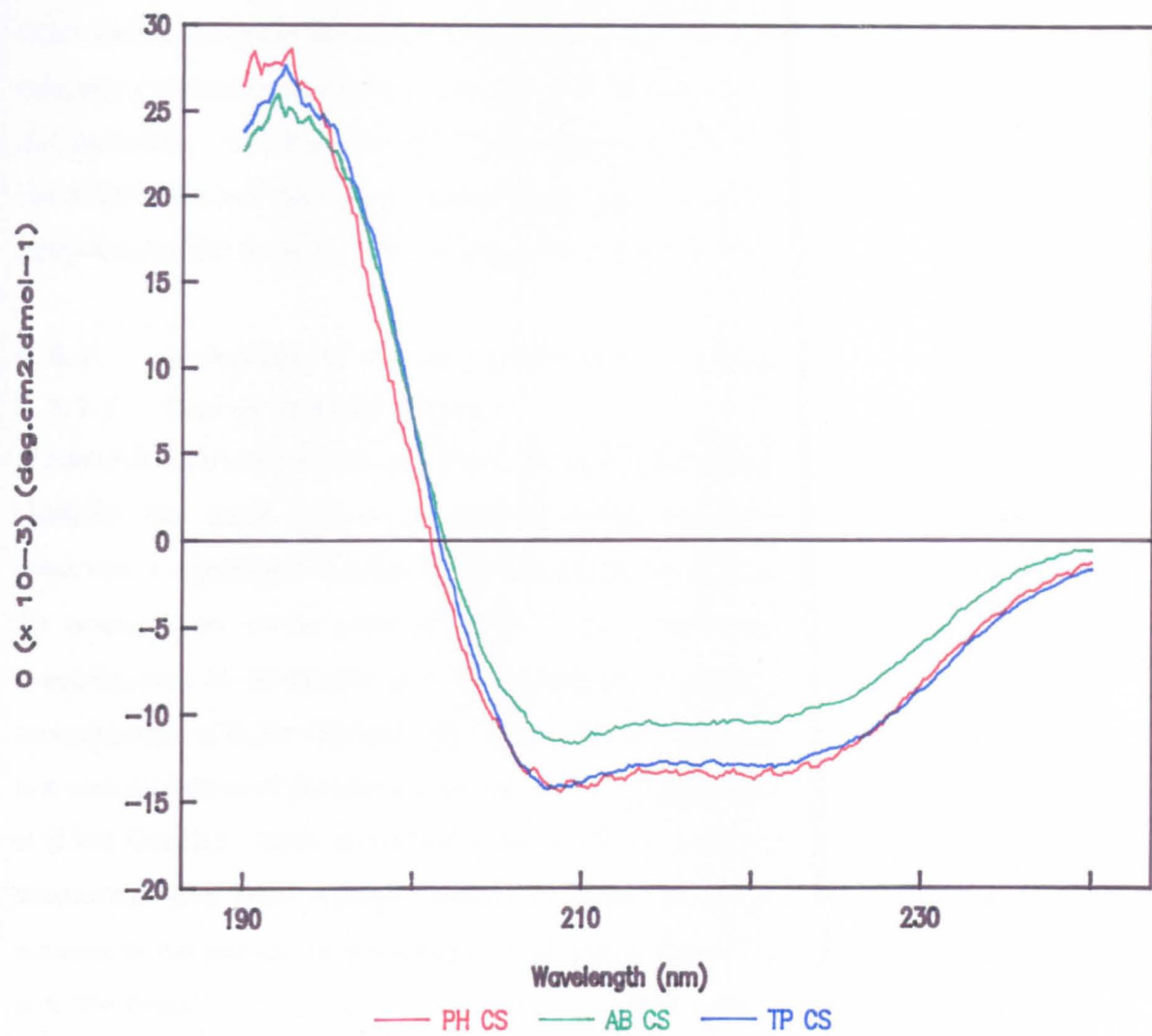


Figure 6.1 Far u.v. c.d. spectra of citrate synthases

Spectra were recorded at 20 °C in 20mM Tris-HCl buffer, pH 8.0.

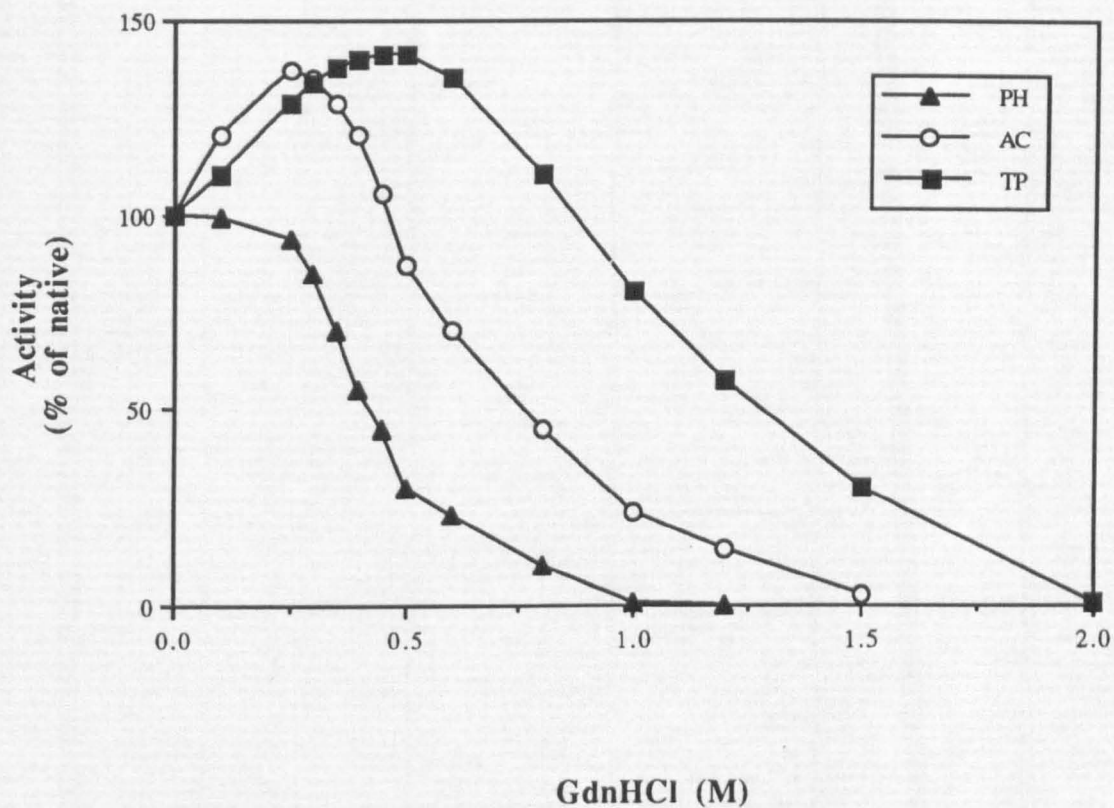


secondary structure analysis program (Provencher and Glöckner, 1981) gave the following estimates of secondary structure for the *Tp. acidophilum* enzyme:  $\alpha$ -helix, 53%;  $\beta$ -sheet, 43%; remainder 4%. These values are similar to those obtained for pig heart citrate synthase (section 5.3.1) and highlight the similarities between the two enzymes in terms of secondary structure. The secondary structure of the enzyme from *Ac. anitratum* was found to differ significantly from that of the other two. Applying the CONTIN procedure to the spectral data gave the following values for secondary structure content:  $\alpha$ -helix, 38%;  $\beta$ -sheet, 60%; remainder 2%.

## 6.3.2 Unfolding of citrate synthases by GdnHCl

### 6.3.2.1 Loss of catalytic activity

Figure 6.2 shows the loss of activity of citrate synthase in the absence and presence of GdnHCl. The results indicate that in the presence of low concentrations of GdnHCl there was a significant increase in the activity of the enzymes from *Ac. anitratum* and *Tp. acidophilum* of the order of  $\leq 40\%$ . In the former this activation was effected at concentrations of denaturant of  $\leq 0.4\text{M}$  while in the latter activation occurred up to a concentration of  $0.7\text{M}$  GdnHCl. By contrast the pig heart enzyme was not activated at low concentrations of denaturant; indeed, the enzyme was almost completely inactivated at  $0.7\text{M}$  GdnHCl. Such increases in the activity of some enzymes in the presence of denaturant have been reported previously. Johnson and Price (1987) noted a 15% increase in the activity of phosphoglycerate mutase from *Schizosaccharomyces pombe* in  $0.5\text{M}$  GdnHCl while Rehder and Jaenicke (1992) reported a 3-fold increase in the activity of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the hyperthermophilic eubacterium *Thermatoga maritima*. It should be noted that the mesophilic enzyme from lobster muscle has been shown (Tsou *et al.*, 1983) to be completely inactivated in  $0.5\text{M}$  GdnHCl. It has been suggested that the increase in activity of certain enzymes could be due to small changes in their conformation which allow greater flexibility or "loosening" of the protein. This flexibility could be important for the conformational changes undergone during the catalytic cycle. Indeed, Tsou (1986) has reported that the active sites of enzymes may be situated in a limited region which is more flexible than the molecule as a whole and, thus, more sensitive to the presence of denaturant at concentrations which do not affect the secondary or tertiary structure of the molecule. In the case of hyperthermophilic GAPDH it was postulated



**Figure 6.2** Loss of activity of citrate synthases in the presence of increasing concentrations of GdnHCl

Samples were incubated for 15 min in 50mM Hepps- $\text{Na}^+$  / 1mM EDTA (pH 8.0), containing 0.1mM dithiothreitol and the indicated concentration of GdnHCl before aliquots were taken for assay. Pig heart (PH) and *Ac. anitratum* (AC) were assayed at 20°C and *Tp. acidophilum* (TP) were assayed at 55°C.

that the loosening effect of the denaturant causes the enzyme to adopt a less rigid conformation which is similar to its mesophilic counterpart (Rehaber and Jaenicke, 1992).

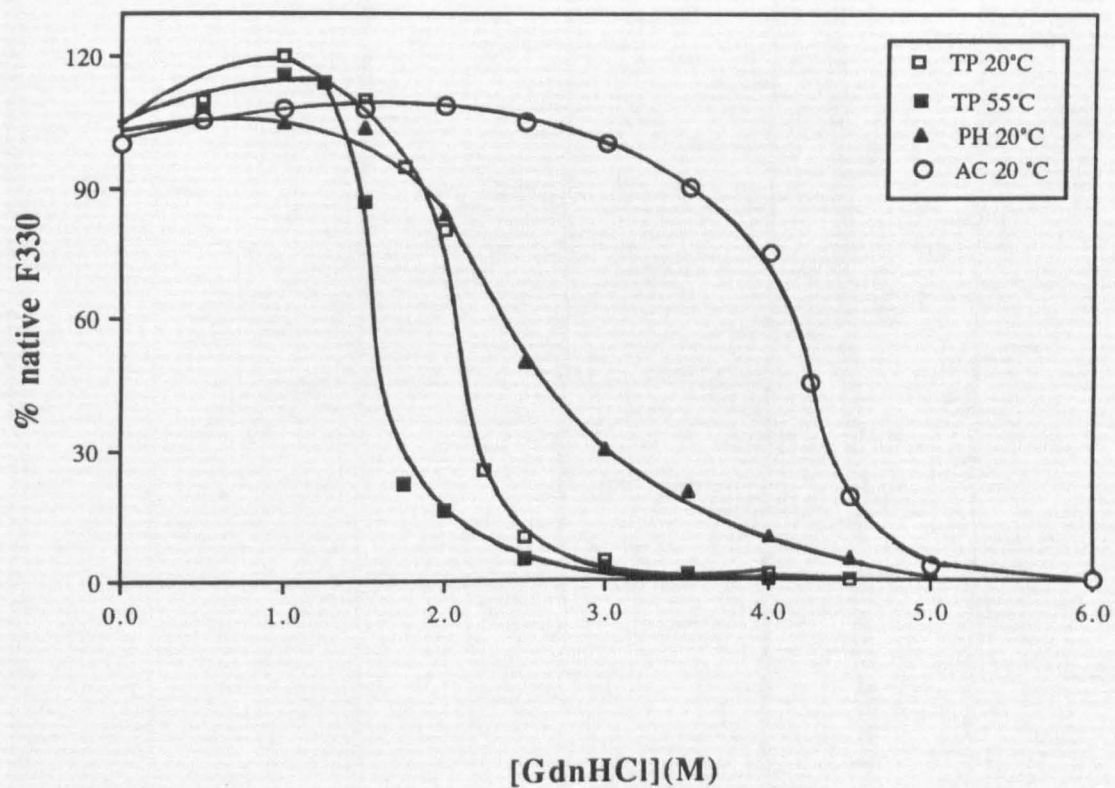
The mid-points for the inactivation, by GdnHCl, of citrate synthases from *T. acidophilum* and *Ac. anitratum* occurred at 1.30M and 0.75M respectively. From these studies it is evident that the active site of the enzyme from *Tp. acidophilum* is most stable towards inactivation by GdnHCl.

#### **6.3.2.2 Changes in tertiary and secondary structure**

Figure 6.3 shows that increases, of up to 20%, in the fluorescence emission of citrate synthases from *Ac. anitratum* and *Tp. acidophilum* were found to occur at low concentrations of GdnHCl ( $\leq 1.5M$ ). This pattern resembles that of the pig heart enzyme and has been taken to reflect the “loosening” of the protein due to the interaction of GdnHCl with amino acid side chains. At concentrations of greater than 1.5M the unfolding patterns of the citrate synthases were found to be markedly different. The enzyme from *Tp. acidophilum* was found to be less stable following incubation (at both 55 °C and 20 °C) than the citrate synthases from pig heart and *Ac. anitratum*. The midpoints of unfolding for the citrate synthases (at 20 °C) were as follows: *Tp. acidophilum*, 1.6M; Pig heart, 2.4M; *Ac. anitratum*, 4.2M. Figure 6.4 shows a similar unfolding pattern for the changes in the secondary structure of these enzymes. In each case it was found that the enzyme from *Ac. anitratum* was the most stable towards GdnHCl-induced unfolding. Given the hexameric structure of this enzyme, it is possible that the interactions between the six subunits confer greater stability on the protein than is found in the dimeric forms of citrate synthase.

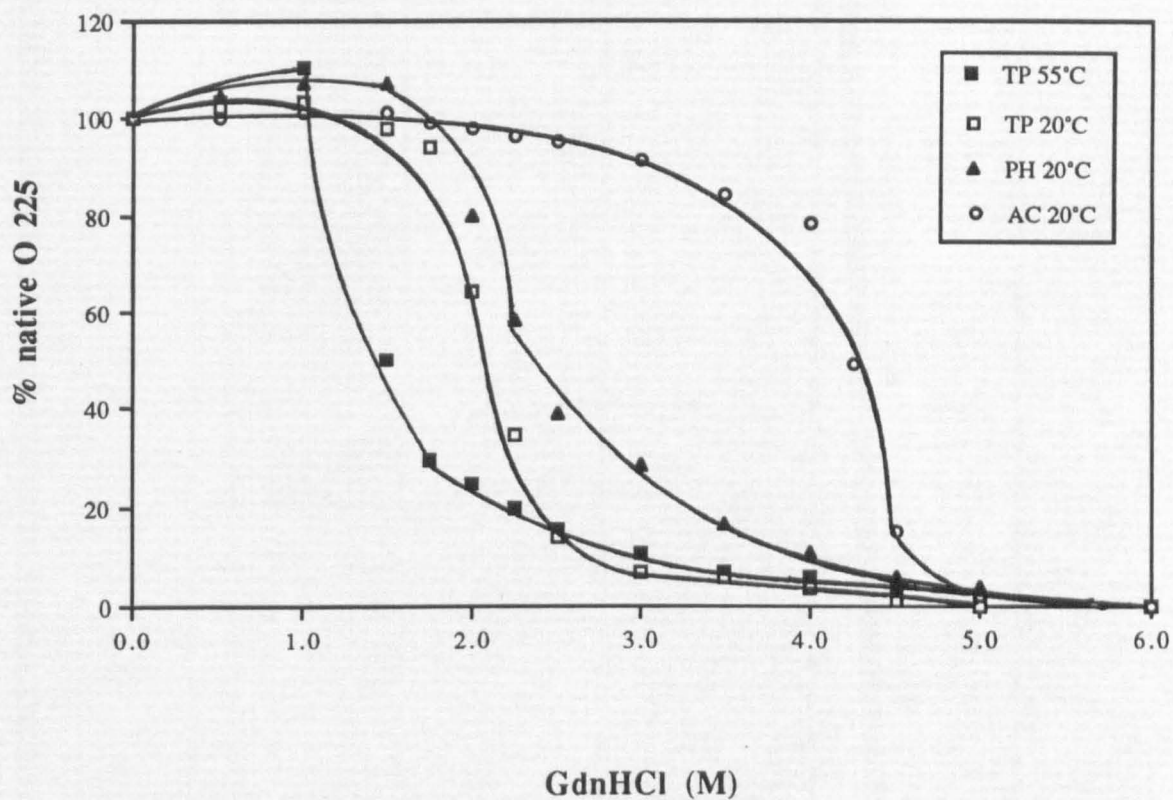
#### **6.3.2.3 Quenching of fluorescence by succinimide**

Succinimide does not readily penetrate the interior of the protein matrix (without local fluctuations in the protein structure) due to size constraints; it is therefore a useful probe of the flexibility of a protein and the relative exposure of tryptophan residues (Edward, 1970; Eftink and Ghiron, 1984). The quenching of fluorescence of the citrate synthases by succinimide was used to assess and compare their protein structure in terms of flexibility and tryptophan exposure in the absence and presence of GdnHCl. It is evident from the Stern-Volmer constants obtained that the intrinsic fluorescence of the



**Figure 6.3** Changes in the fluorescence emission at 330nm of citrate synthases in the presence of GdnHCl

Enzyme samples from pig heart (PH), *Ac. anitratum* (AC) and *Tp. acidophilum* (TP) were incubated for 15 min in 50mM Hepps-Na<sup>+</sup> / 1mM EDTA (pH 8.0), containing 0.1mM dithiothreitol and the indicated concentrations of GdnHCl before changes in F<sub>330</sub> were determined.



**Figure 6.4** Changes in ellipticity at 225nm of citrate synthases in the presence of GdnHCl

Conditions employed were as described in Figure 6.3

pig heart enzyme is quenched to a greater extent by succinimide ( $1.68\text{M}^{-1}$ ) than the enzymes from *Tp. acidophilum* and *Ac. anitratum* ( $0.53\text{M}^{-1}$  and  $0.45\text{M}^{-1}$  respectively) presumably reflecting the greater flexibility of pig heart citrate synthase compared with the other enzymes. These studies also show that in the presence of 6M GdnHCl similar Stern-Volmer constants  $3.1\text{M}^{-1}$ ,  $3.5\text{M}^{-1}$  and  $2.8\text{M}^{-1}$  were obtained for the citrate synthases from pig heart *Ac. anitratum* and *Tp. acidophilum* respectively, indicating the increased accessibility of the proteins to quencher in the unfolded state.

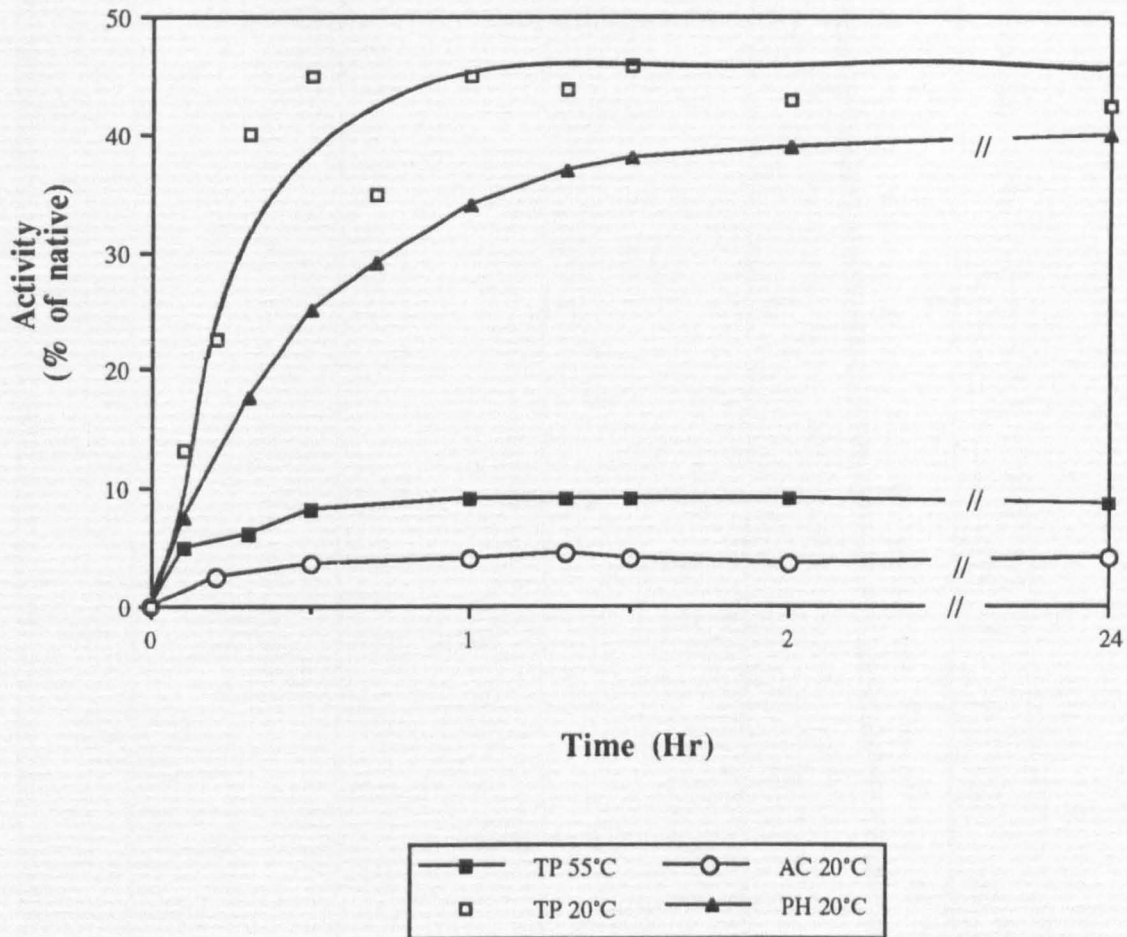
The results of the quenching studies offer a possible explanation for the observed activation of citrate synthases from *Ac. anitratum* and *Tp. acidophilum* in the presence of low concentrations of denaturant. At 20 °C these enzymes are in a more rigid conformation than their mesophilic counterpart from pig heart. The “loosening” effect of the denaturant, at low concentrations, may allow the proteins to adopt a more flexible conformation which is comparable with the mesophilic enzyme and leads to an increase in their specific activities. In future work the succinimide quenching of citrate synthases, in the presence of low concentrations of GdnHCl, should be studied to examine this proposal.

### **6.3.3 Reactivation of citrate synthases following GdnHCl-induced denaturation**

The reactivation of the enzymes following incubation for a period of 15 min in denaturant was attempted by dilution into or dialysis against two changes of buffer. In each case the residual concentration of GdnHCl was less than 0.02M as judged by refractive index measurements.

#### **6.3.3.1 Reactivation by dilution of denaturant**

Figure 6.5 and Table 6.1 show that substantial recovery of the catalytic activity of the enzyme from *Tp. acidophilum* can be obtained following the dilution of GdnHCl with buffer. Most of the activity was regained within the first hour following dilution. The extent of reactivation was influenced by the buffer system used, the temperature at which reactivation was initiated, the concentration of protein, and the inclusion of dithiothreitol and/or BSA in the reactivation mix. Optimal reactivation (approximately 55-60%) was achieved at 20 °C when the final enzyme concentration was approximately



**Figure 6.5** Reactivation of citrate synthases following GdnHCl-induced denaturation and subsequent dilution

Citrate synthases from pig heart (PH) *Ac. nitratum* (AC) and *Tp. acidophilum* (TP) were incubated for 15 min in 50mM Hepps-Na<sup>+</sup> / 1mM EDTA (pH 8.0), in the presence of 6M GdnHCl prior to dilution. Samples were assayed for citrate synthase activity after incubation at 20°C or 55°C as indicated.

**Table 6.1** Conditions affecting the regain of activity of pig heart citrate synthases following dilution or dialysis of denaturant; PH, TP and AC refer to enzymes from pig heart, *Tp acidophilum* and *Ac anitratum* respectively, incubated at the indicated temperatures

Refolding conditions	Regain of activity (%) <sup>≈</sup>			
	TP 20 °C	TP 55 °C	AC 20 °C	PH 20 °C
<u>By dilution into:</u> <sup>f</sup>				
20mM TrisHCl 1mM EDTA (pH 8.0)	<1	< 1	< 1	<1
20mM TrisHCl 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol	45	7	4	42
20mM TrisHCl 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol + BSA (1mg/ml)	50	8	4	46
50mM Hepps 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol	51	10	3.5	52
50mM Hepps 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol + BSA (1mg/ml)	57	10	4	55
<u>By dialysis into:</u> <sup>†</sup>				
20mM TrisHCl 1mM EDTA (pH 8.0)	< 1	< 1	< 1	< 1
20mM TrisHCl 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol	60	10	< 2	59
50mM Hepps 1mM EDTA (pH 8.0) + 0.1mM dithiothreitol	46	8	< 2	44

**notes:**

≈ - All experiments were carried out in triplicate and the activity data shown represent the mean values obtained

f - Concentration of enzyme following dilution was approximately 5µg/ml

† - Concentration of enzyme following dialysis was approximately 10µg/ml



5µg/ml and the reactivation buffer was 50mM Hepps-Na, pH 8.0, containing 1mM EDTA 0.1mM dithiothreitol and BSA at a concentration of 1mg/ml. These results are similar to those obtained for the pig heart enzyme (section 5.3.3). When reactivation by dilution was performed at 55 °C, the optimal growth temperature of *Tp. acidophilum*, it was found that only 10% of the enzyme activity could be recovered using the same refolding conditions described above. It is possible that this is due to the instability of transient intermediates in the folding pathway or that the folding protein is trapped in a particular conformation which is favoured at the higher temperature.

Less than 5% reactivation of citrate synthase from *Ac. anitratum* could be achieved following dilution of denaturant (Figure 6.5). The addition of BSA did not result in any detectable enhancement in catalytic activity. It is probable, given the hexameric structure of this enzyme, that the refolding pathway is more complex than that of the dimeric enzyme with an increased susceptibility to the formation of incorrect folding intermediates or the wrong association of subunits. Glutamate dehydrogenase is an example of another hexameric protein which has, under the experimental conditions employed, been shown to be unable to refold into a functional enzyme *in vitro* following denaturation and subsequent dilution (Muller and Jaenicke, 1980; West and Price, 1988; Kelly and Price, unpublished observations).

#### 6.3.3.2 Reactivation by dialysis of denaturant

When reactivation of the citrate synthases was initiated by dialysis, the recovery of activity of the enzyme from *Tp. acidophilum* was broadly similar to that obtained following the dilution procedure in the presence of BSA (Table 6.1). At a final concentration of enzyme of approximately 10µg/ml the recovery of activity following dialysis, against 20mM Tris-HCl containing 0.1mM dithiothreitol and 1mM EDTA, was in the range from 55-60%. The results obtained following dialysis in Hepps buffer gave slightly lower recoveries of activity. Unlike the pig heart (section 5.3.3.2) and *Tp. acidophilum* enzymes the reactivation of the *Ac. anitratum* citrate synthase could not be achieved using the dialysis procedure.

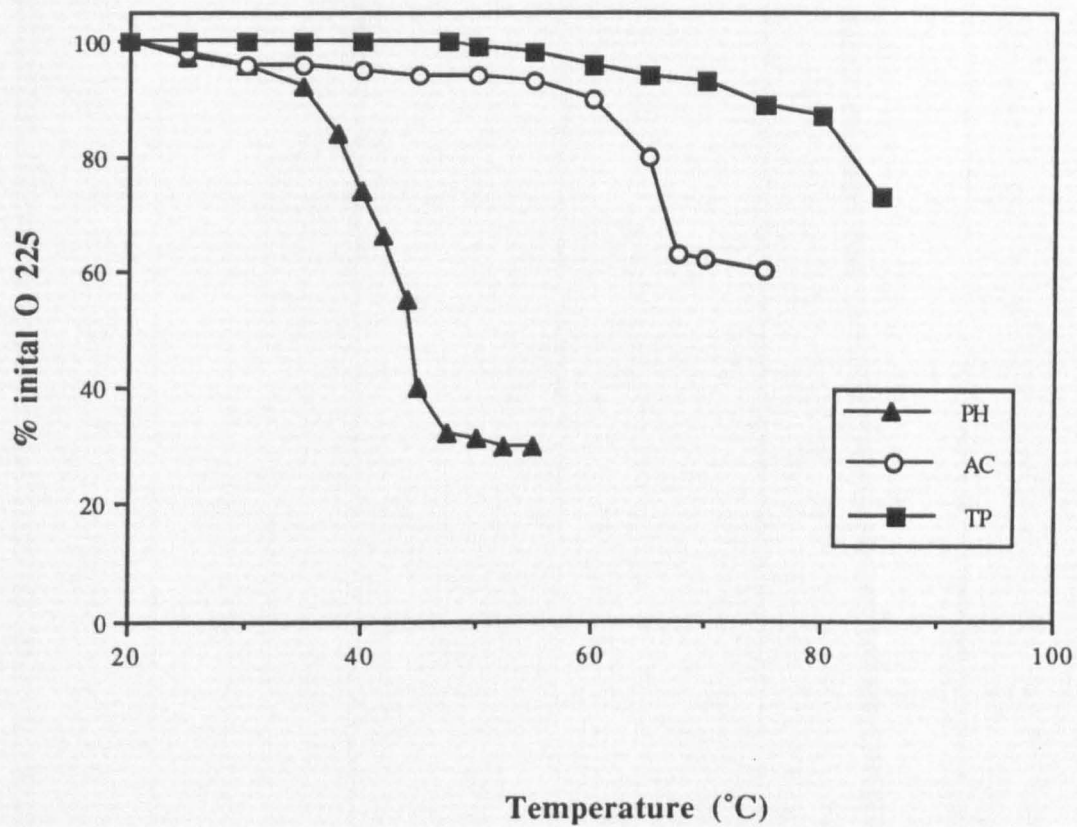
### 6.3.4 Thermal unfolding and attempted refolding of citrate synthases

#### 6.3.4.1 Loss of catalytic activity

Thermal inactivation studies were performed by Dr David W. Hough of the University of Bath. It was found that citrate synthase from pig heart was progressively inactivated at temperatures in the range 42 °C - 50 °C. The enzymes from *Ac. anitratum* and *Tp. acidophilum* were found to be markedly more resistant to thermal inactivation showing rapid inactivation at temperatures in the ranges 60 °C - 70 °C and 74 °C - 84 °C respectively.

#### 6.3.4.2 Changes in secondary structure

Figure 6.6 shows the changes in the ellipticity of the three citrate synthases as a function of temperature. These data show that the mid-point values, relating to loss of secondary structure, for pig heart and *Ac. anitratum* enzymes occurs at 45 °C and 65 °C respectively. These results parallel the thermal inactivation data. At least 90% of the native ellipticity at 225nm of the *Tp. acidophilum* enzyme remains following incubation at approximately 70 °C (Figure 6.6). Although it was difficult to determine the end-point of thermal unfolding of this thermophilic enzyme (due to difficulties in maintaining constant temperatures in the cuvette above 85 °C) it was observed that prolonged incubation at temperatures above 80 °C resulted in the precipitation of protein. This effect was also observed with the pig heart enzyme at temperatures beyond 45 °C. By contrast, precipitation was not evident when the *Ac. anitratum* citrate synthase was incubated at temperatures greater than 70 °C, although subsequent cooling to 20 °C, for up to 12 hr, did not result in any significant increase in ellipticity at 225nm. The results obtained by Dr Alan Cooper (University of Glasgow) using differential scanning calorimetry (d.s.c) were in reasonable agreement with the thermal unfolding data obtained by c.d. The apparent  $T_m$  for the enzymes from pig heart, *Ac anitratum* and *Tp. acidophilum* were 48.3 °C, 67.5 °C and 85.3 °C respectively. The asymmetry of the traces of the pig heart and *Tp. acidophilum* enzymes were taken to reflect the precipitation of these proteins at temperatures above the thermal transition region. By contrast no such asymmetry was evident in the trace obtained for the enzyme from *Ac. anitratum*. A second cycle of d.s.c. was performed on the latter citrate synthase after cooling to 20 °C however no transition could be detected indicating that the cooling process did not reverse the thermal denaturation of this enzyme. These results imply that



**Figure 6.6 Thermal unfolding of citrate synthases as monitored by changes in ellipticity at 225nm**

Citrate synthase samples from pig heart (PH), *Ac. anitratum* (AC) and *Tp acidophilum* (TP) in 50mM Hepps- $\text{Na}^+$  / 1mM EDTA were heated over the indicated temperature range and at each temperature the average of 10 values of  $\theta_{225}$  was recorded.

the thermal unfolding of citrate synthases from all three sources, under the experimental conditions employed is an irreversible process. It is noteworthy that the reactivation of adenylate kinase (from rabbit muscle) following thermal denaturation also leads to precipitation of the enzyme which is not reversible on cooling and incubation for a prolonged period of time at 4 °C (Zhang *et al.*, 1993). However, these workers have reported that 80% reactivation of the enzyme could be achieved by cooling and incubating the enzyme in 3M GdnHCl followed by dilution of the denaturant. It was observed that in the presence of GdnHCl disaggregation occurred. In future studies it would be interesting to establish whether incubation of the thermally inactivated citrate synthases in GdnHCl prior to the dilution step would yield active enzyme.

## 6.4 Summary of Results

The differential stabilities of citrate synthases from three diverse sources have been examined towards thermal and chemical denaturation. It was found that the enzymes from *Tp. acidophilum* and pig heart were markedly similar in terms of secondary structure content, despite their low sequence identity (20%). By contrast the secondary structure of enzyme from *Ac. anitratum* was found to differ significantly from the other two. The citrate synthase from pig heart was found to lose activity in the presence of low concentrations of GdnHCl (in the range from 0.25M to 1M) whereas citrate synthases from *Ac. anitratum* and *Tp. acidophilum* were activated at concentrations of GdnHCl  $\leq 0.4\text{M}$  and  $\leq 0.7\text{M}$ , respectively. Succinimide quenching studies revealed that the latter citrate synthases are more rigid than the pig heart enzyme in the absence of denaturant. The denaturant (at concentrations of up to 1.5M) was found to increase the values obtained for the fluorescence emission (at 330nm) and ellipticity (at 225nm) of all three citrate synthases. It is possible that the apparent increases in tertiary and secondary structure reflect the interactions of low concentrations of GdnHCl with amino acid side chains and/or solvent which, by altering the solute/solvent interactions may allow additional structural elements to form. At higher concentrations of denaturant (greater than 1.5M) the thermophilic enzyme was least stable towards GdnHCl denaturation while, the hexameric enzyme was most stable in GdnHCl compared with the other citrate synthases. In thermal studies it was shown that the thermophilic enzyme was more stable towards thermal denaturation than the citrate synthase from *Ac. anitratum* which, in turn, was more stable than the pig heart enzyme.

Reactivation studies following dialysis or dilution of denaturant revealed that moderate recoveries of activity could be obtained for the citrate synthases from both pig heart (section 5.3.3) and *Tp. acidophilum* depending on the refolding conditions employed. Less than 5% recovery of activity was achieved by the hexameric citrate synthase using the optimal refolding conditions required for the reactivation of the other enzymes. Given the higher number of subunits in this enzyme it is not surprising that reactivation is more difficult to effect. The reactivation of the citrate synthases following thermal unfolding and subsequent cooling for a prolonged period of time could not be achieved under the experimental conditions employed.

*Chapter seven*

**A Preliminary Study of the Unfolding and attempted  
Refolding of the Bacterial Chaperone  
Protein GroEL (cpn 60)**

## 7.1 Introduction

The polypeptides GroEL,  $M_r$  57 200 and GroES,  $M_r$  10 300 are encoded in the GroE operon of *Escherichia coli* and belong to a class of proteins called molecular chaperones which have been implicated in the post translational folding and transport of proteins within a cell (Ellis, 1987, also section 1.7) The GroE gene products have been assigned to a sub-class of chaperones, named chaperonins, which exhibit a considerable degree of sequence identity and which share similar structural and functional characteristics. Examples of other chaperonins include: hsp60 and hsp10 from yeast mitochondria; Rubisco subunit binding protein and Cpn10 from plant chloroplasts (Hemmingsen *et al.*, 1988; Ellis and Van Der Vies, 1991). Following the observation that GroEL is involved in mediating protein folding by acting as a molecular chaperone there has been a growing interest in the mechanism of action of this chaperonin and its structural and biochemical characterisation. The fact that several other chaperonins which have been isolated possess a high percentage (43-54%) of sequence identity to the GroEL protein (Hemmingsen *et al.*, 1988) has resulted in speculation that the chaperonins share a common function in assisting protein folding and most probably have a similar mode of action.

The discovery that GroEL is required for the assembly of the  $\lambda$ , T4 and T5 phages was noted over 20 years ago (Georgopoulos *et al.*, 1972, 1973; Zweig and Cummings, 1973). Subsequent studies revealed that both GroEL and GroES are induced during the cellular response to heat shock (for which reason it is often referred to as a member of the hsp60 family) and are also required for cell viability under normal growth conditions (Fayet *et al.*, 1989). In 1979 Hendrix purified and examined the properties of the GroEL protein (Hendrix, 1979). He suggested from biophysical studies that GroEL comprises 14 identical subunits. Studies using electron microscopy led to the conclusion that the oligomeric GroEL complex (viewed from above) consists of two heptameric rings arranged, one on top of the other, to give rise to a cylindrical structure with 7-fold symmetry, a diameter of 125 Å and a height of 100 Å (Hendrix, 1979;

Hohn *et al.*, 1979). It was also noted during these early investigations that the GroEL complex was capable of hydrolysing ATP at a low rate. These findings have since been confirmed and elaborated by several groups in an attempt to elucidate the interactions of GroEL with GroES and polypeptide substrates. Indeed such studies have shown that GroEL can bind “promiscuously” to many unfolded polypeptides and that GroES and the hydrolysis of ATP are involved in the sequence of events leading to the correct folding or folding-competent state of the particular protein. Investigations carried out by Goloubinoff *et al.* (1989) and Viitanen *et al.* (1990) on the refolding of denatured Rubisco from *Rhodospirillum rubrum* have shown that the folding intermediate(s) of Rubisco form(s) a stable binary complex with GroEL. In other words, the chaperonin binds nonnative protein. These studies also demonstrated that GroES in the presence of MgATP and K<sup>+</sup> ions triggers the release of the polypeptide from the binary complex with GroEL. A lag phase in the regain of activity of Rubisco following its release suggested that the association of the dimeric Rubisco occurs in solution rather than in the complex with GroEL. Extending these investigations Viitanen *et al.* (1992) demonstrated the existence of the chaperonin complex with Rubisco (radioactively-labelled with [<sup>35</sup>S] methionine) which had been previously denatured in 5M GdnHCl and then diluted 50-fold into a solution containing GroEL. The mixture containing Rubisco and GroEL was then applied to an HPLC-TSK, gel permeation, sizing column which showed that approximately 75% of the radioactivity eluted with the fraction corresponding to that of the GroEL. When this experiment was repeated using native [<sup>35</sup>S] Rubisco the formation of a binary complex with GroEL could not be detected. The inclusion of MgATP and GroES 1min after dilution of the denaturant with a solution containing a molar excess of GroEL was examined as described above. It was found that there was a substantial decrease in the radioactivity eluted in the fraction corresponding to GroEL. In addition a major and a minor peak were observed corresponding to those of the native Rubisco dimer (enzymatically active) and the inactive folded dimer respectively. In the absence of GroES there was a marked reduction (approximately 50%) in the radioactivity found in the binary complex fraction and no new peaks were observed indicating that MgATP is capable of reducing the affinity of GroEL for nonnative Rubisco but this process does not promote the folding of the protein. These results imply that GroES is required to assist in the promotion of a



folding-competent state in Rubisco such that the susceptibility of the protein to form insoluble aggregates upon release from the chaperonin complex is less favourable. GroEL has been shown to interact with a host of other proteins to form a binary complex and assist protein folding. Examples include: pre- $\beta$ -lactamase (Laminet *et al.*, 1990); GroEL (Lissin *et al.*, 1990); citrate synthase (Buchner *et al.*, 1991); rhodanese, (Martin *et al.*, 1991; Mendoza *et al.*, 1991); dihydrofolate reductase (DHFR) (Martin *et al.*, 1991). Studies on these proteins suggested that GroEL is able to bind 1-2 molecules of protein folding intermediate per GroEL 14-mer.

Several groups have sought to determine the way in which GroEL recognises a wide variety of proteins in their nonnative state. Langer *et al.* (1992) noted the apparent inability of GroEL to recognise an extended polypeptide chain. They concluded that the presence of secondary structural elements are required for substrate binding to GroEL. Whether or not GroEL recognises a particular secondary structure motif remains in question since it has been demonstrated that it binds proteins which have the propensity to form either  $\alpha$ -helical structures or  $\beta$ -sheet structures (Landry and Gierasch, 1991; Schmidt and Buchner, 1992). The fact that GroEL is unable to bind native proteins suggests that hydrophobic surfaces might be the preferred sites of recognition since they are often inaccessible in the native state but tend to be exposed in the nonnative conformations. The conformation of the protein substrates DHFR and rhodanese individually bound to GroEL has been examined by monitoring the intrinsic fluorescence of the binary complex, ANS binding studies and the susceptibility of the substrate proteins to proteases (Martin *et al.*, 1991). These studies showed that the tryptophan residues of the bound substrate proteins were partially exposed, the proteins were susceptible to proteolytic digestion at low concentrations of protease and that binding of ANS occurred indicating the accessibility of hydrophobic residues to solvent. The nature of these findings suggest that GroEL could possibly stabilise proteins in a conformation similar to that of the "molten globule" prior to release and subsequent folding to their native conformations.

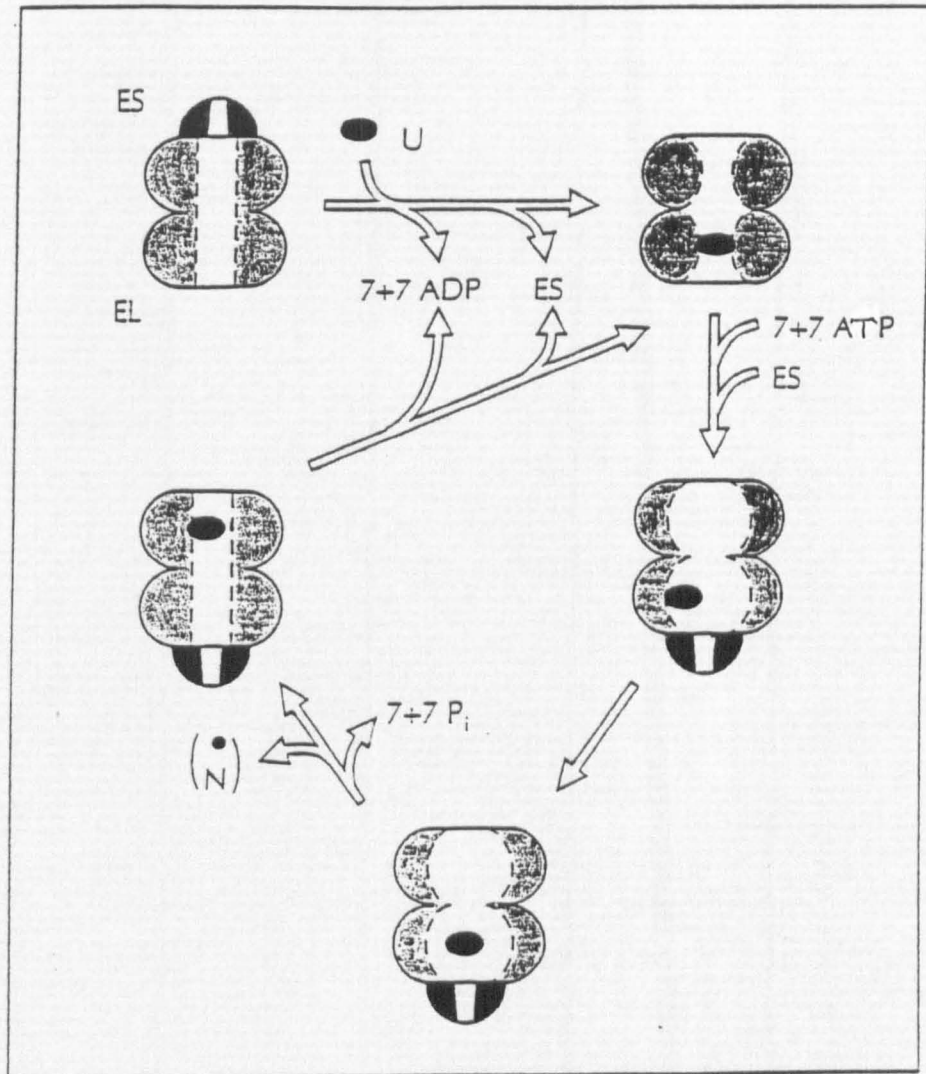
The location of the site(s) at which substrate proteins bind to GroEL has also generated much recent interest over the last few years. Several lines of evidence now exist which suggest that the substrate polypeptides are bound in the central cavity of the GroEL

molecule. Langer *et al.* (1992) showed, using electron microscopic image analysis and biochemical studies, that the binding of denatured polypeptide to GroEL results in an observed increase in the protein density in the central cavity. These workers also showed that the binding of GroES to one end of GroEL results in conformational changes at both the proximal and distal ends of the complex. They demonstrated that the asymmetric interaction between GroEL and GroES protects one half of the GroEL particle from cleavage of 50 carboxy-terminal residues by proteinase K.

Braig *et al.* (1993) provided further insight into the binding of polypeptide chains by GroEL using small gold particles covalently bound to unfolded chicken DHFR. Denaturation of the gold-DHFR species and subsequent dilution into buffer containing GroEL yielded chaperonin-polypeptide complexes which could be examined by electron microscopy. In these experiments it was shown that gold densities were observed in the central cavity of the particle and that in some cases two gold particles were associated with the same GroEL.

Martin *et al.* (1993) have proposed that GroEL assists protein folding in a cycle of interactions between GroEL, GroES, non-native protein and MgATP. In this cyclic process (summarised in Diagram 7.1) GroEL which is complexed to GroES and ADP binds protein in a partially folded state, causing the release of GroES and the exchange of ADP for ATP. The hydrolysis of ATP, stimulated by the bound protein and the re-binding of GroES, causes the release of the bound protein allowing it to fold in the central cavity of the GroEL complex. If complete folding occurs the protein is released from the central cavity of the GroEL-GroES-ADP complex. The latter is free to bind other partially folded proteins. If complete folding does not occur the protein in the central cavity re-binds to the GroEL-GroES-ADP complex and the cycle of events continues.

To date, a high resolution, three dimensional structure of GroEL is not available. Crystallisation and preliminary X-ray studies of GroEL have been conducted (Spangfort *et al.*, 1993) however, the crystals obtained were found to diffract to a maximum of only 7 Å. Recent X-ray analysis of these crystals (Svenssen *et al.*, 1994) suggests that the GroEL oligomer has one internal 7-fold symmetry axis which is consistent with the



**Diagram 7.1 A model for the reaction of GroEL and GroES chaperonin-assisted protein folding**

For description of model see text. U and N represent partially and fully folded protein respectively. EL and ES refer to GroEL and GroES respectively. (The model has been adapted from Martin *et al.*, 1993).

assembly of seven GroEL subunits into a heptamer and seven two-fold axes perpendicular to the heptamer indicating the probable head to head arrangement of the two heptamers to form native GroEL.

Lissin *et al.* (1990) have proposed that functional GroEL particles are involved in the assembly of GroEL monomers in a mechanism known as “self chaperoning”. In view of this proposal, the important role played by GroEL in the folding and assembly of proteins and the complex structure of this chaperonin, it was considered of interest to examine the unfolding and refolding properties of GroEL in some detail.

Some of the work described in this chapter was jointly undertaken with Graeme J. Thomson of the University of Glasgow.

## **7.2 Materials and Methods**

### **7.2.1 Materials**

GroEL purified from *E. coli* strain MC1061 was kindly provided by Drs Stephen Wood and Arlene auf der Mauer of the Department of Crystallography, Birkbeck College, Malet Street, London, England).

Graeme J. Thomson (University of Glasgow) purified GroEL from *E. coli* strain DH1pND5 which was kindly gifted by Drs N MacLennan and M. Masters (Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, Scotland).

ANS (hemimagnesium salt) was purchased from Sigma, Poole, U.K.

### **7.2.2 Methods**

#### **7.2.2.1 Determination of purity of GroEL**

The purity of GroEL isolated from *E. coli* strains MC1061 (carrying the plasmid pND5) and DH1pND5 were examined by Arlene auf der Mauer and Graeme J. Thomson respectively, using gel filtration on Superose 6 and anion exchange chromatography at pH 7.5 on MonoQ. In each case the protein was found to elute from both of these columns as a single symmetrical peak. Coomassie blue staining following SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels indicated that at least 98% of the material migrated as a single peak with an apparent  $M_r$  of  $57\,000 \pm 2000$ .

#### **7.2.2.2 Determination of protein concentration**

The protein concentration of GroEL was determined using a value of 0.285 corresponding to the extinction coefficient of a 1mg/ml solution at 280nm (Price *et al.*, 1991).

The Coomassie blue binding assay (Sedmak and Grossberg, 1977) using bovine serum albumin as a protein standard, was also employed as described earlier (section 2.2.1). The values obtained by this method were found to be within 10% of those obtained spectrophotometrically at 280nm.

### 7.2.2.3 Assay for ATPase activity

The ATPase activity of GroEL was measured in collaboration with Graeme J. Thomson using a coupled assay procedure involving pyruvate kinase and lactate dehydrogenase (Price *et al.*, 1993).

### 7.2.2.4 Activity of GroEL in the presence of GdnHCl

The effect of GdnHCl on the ATPase activity of GroEL was monitored using a “coupled quench” assay procedure as described by Price *et al.* (1993).

### 7.2.2.5 Light scattering

Light scattering measurements at 360nm were performed at 20 °C on a Perkin Elmer LS50B spectrofluorimeter and analysed according to Parr and Hammes (1975) and Tashiro *et al.* (1982). GroEL samples were filtered through a 0.2µM pore-size membrane, prior to measurement, to ensure a dust free solution. Samples containing GroEL in the absence and presence of GdnHCl were measured in addition to the relevant buffer controls.

The ratio of molecular weight in the presence of GdnHCl,  $M_i$ , to that in the absence of GdnHCl,  $M_o$ , were determined from the following equation:

$$\frac{M_i}{M_o} = \frac{(n_o)^2 * (\delta n / \delta c_o) \mu^2 * (c_o) * (I_{i,90})}{(n_i)^2 * (\delta n / \delta c_i) \mu^2 * (c_i) * (I_{o,90})}$$

$n$  = refractive index;  $(\delta n / \delta c) \mu$  = refractive index increment at constant chemical potential;  $c$  = enzyme concentration;  $I_{90}$  = scattered light intensity at 90 °C

In the absence of GdnHCl  $n = 1.333$  which is equivalent to the refractive index of water. In the presence of GdnHCl  $n = 1.333 + \Delta n_i$ .

$\Delta n_i$  values were as determined by Nozaki (1972). The values of  $(\delta n / \delta c) \mu$  were as determined by Noelken and Timasheff (1967).

#### **7.2.2.6 Examination of tertiary structure of GroEL**

Near u.v. circular dichroism measurements of GroEL (0.75mg/ml) in the presence and absence of 2M GdnHCl were recorded at 20 °C in a cell of pathlength 0.5cm.

#### **7.2.2.7 Estimation of secondary structure of GroEL**

The secondary structure content of GroEL was estimated using the data obtained from far u.v. circular dichroism studies. The methods of Provencher and Glöckner (1981), Chang *et al.* (1978) and Seigel *et al.* (1980) were employed to estimate the secondary structural elements of the protein (Appendix 1). The spectrum of GroEL (0.25mg/ml) in 50mM potassium phosphate pH 7.5 was recorded in a 0.02cm pathlength cell.

#### **7.2.2.8 Unfolding of GroEL**

The unfolding of GroEL was achieved following incubation in GdnHCl as described previously (section 2.2.3). The extent of unfolding was monitored by changes in catalytic activity, light scattering, fluorescence and circular dichroism. Some preliminary studies on the unfolding of GroEL by urea were also undertaken (see section 7.3.2.4).

#### **7.2.2.9 ANS binding studies**

The binding of ANS was measured as described in section 4.2.2.8. GroEL samples (50µg/ml) were incubated for 15min at 20 °C in the buffer used by Lissin *et al.* (1990) namely, 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate, containing 0.1mM dithiothreitol (pH 7.5).

#### **7.2.2.10 Refolding of GroEL**

The refolding of chaperonin was attempted by dialysing the denatured protein (at 20 °C) against 2 changes of 200 volumes of buffer to remove the GdnHCl. Following dialysis the residual concentration of GdnHCl was found to be less than 0.01M as measured by the refractive index (Nozaki, 1972). The extent of refolding was monitored by changes in activity, fluorescence and circular dichroism.

## 7.3 Results and Discussion

### 7.3.1 Secondary structure of GroEL

The far u.v. spectrum of GroEL is shown in Figure 7.1. The spectrum has the characteristic double minima attributed to proteins containing a substantial amount of  $\alpha$ -helical structure. The data from this spectrum were analysed by the CONTIN procedure of Provencher and Glöckner (1981) over the range 190nm to 240nm. The estimations of secondary structure were as follows:  $\alpha$ -helix,  $44 \pm 1\%$ ;  $\beta$ -sheet,  $29 \pm 2\%$ ; remainder,  $27 \pm 2\%$ . The methods of Chang *et al.* (1978) and Seigel *et al.* (1980) were also used for comparison. The former gave values for  $\alpha$ -helix,  $\beta$ -sheet and remainder of 48%, 37% and 15% respectively. The  $\alpha$ -helical content of GroEL as determined by the method of Seigel *et al.* (1980) was estimated at 58%. Lissin *et al.* (1990) also obtained a similar value (57%) for helical content of GroEL using the CONTIN procedure. The difficulty in determining an accurate estimation of the protein concentration of GroEL has been noted previously (Price *et al.*, 1991). Given the critical importance of the accurate determination of protein concentration in obtaining reliable estimates of secondary structure it is not surprising that this discrepancy exists. It can be assumed that the helical content of GroEL is in the range from 44% to 58%.

### 7.3.2 Unfolding of GroEL

#### 7.3.2.1 Loss of ATPase activity

The changes in the ATPase activity of GroEL in the presence of increasing concentrations of GdnHCl in the range from 0M to 2M are shown in Figure 7.2. It can be seen that the mid-point of activity loss occurred at approximately 0.35M and that no activity remained above 0.75M GdnHCl. The loss of ATPase activity of GroEL is another example of the high sensitivity of an enzyme to the presence of low concentrations of denaturant. The sensitivity of the active site of fumarase (section 3.3.2.1), NAD<sup>+</sup>-dependent isocitrate dehydrogenase (section 4.3.2.1) and citrate synthase (section 5.3.2.1) have already been noted. The possible interactions of denaturant with the active sites of enzymes will be discussed in section 8.1.3.

#### 7.3.2.2 Changes in light scattering

Figure 7.3 shows the change in  $M_r$  of GroEL (monitored by changes in the intensity of



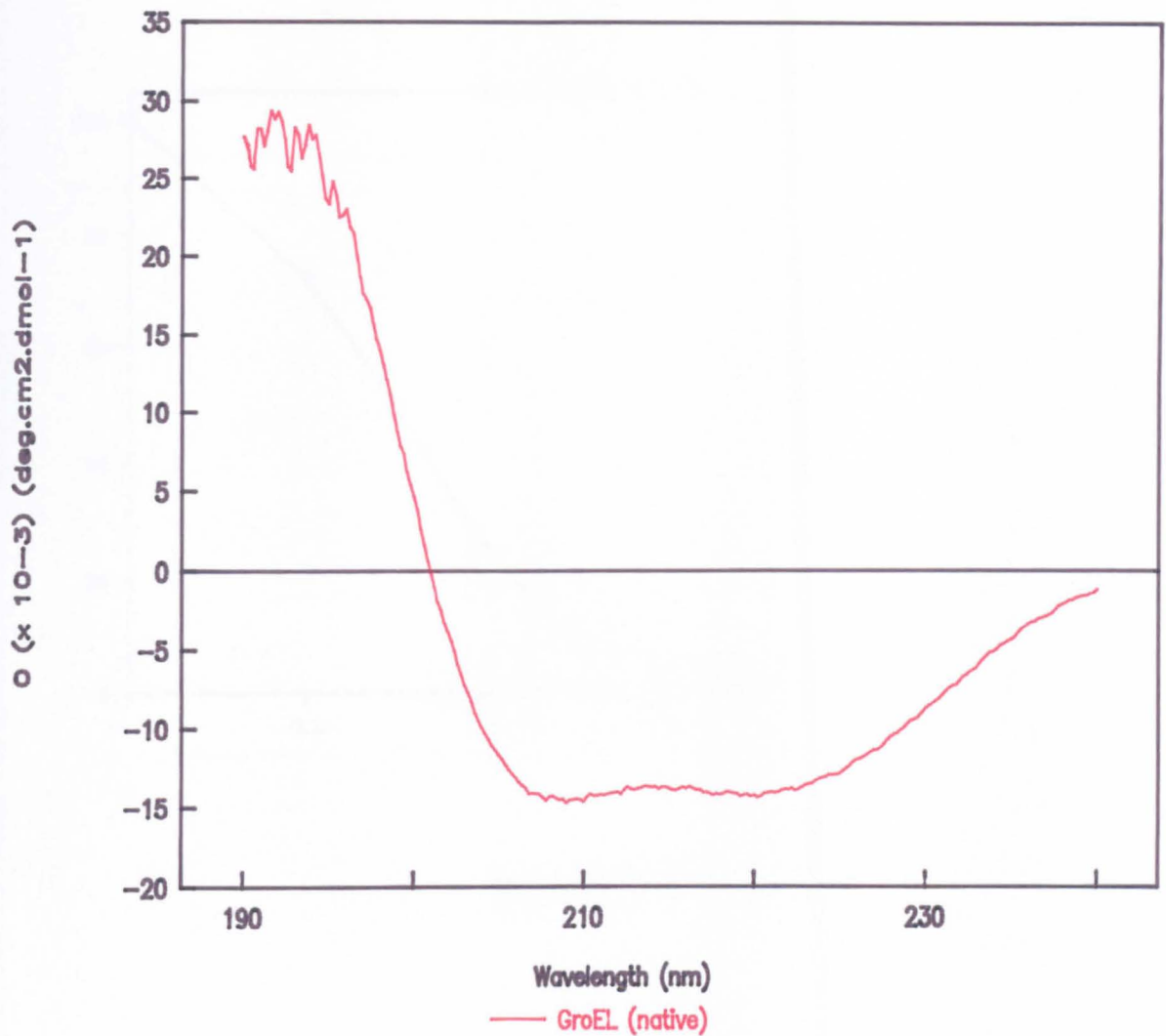
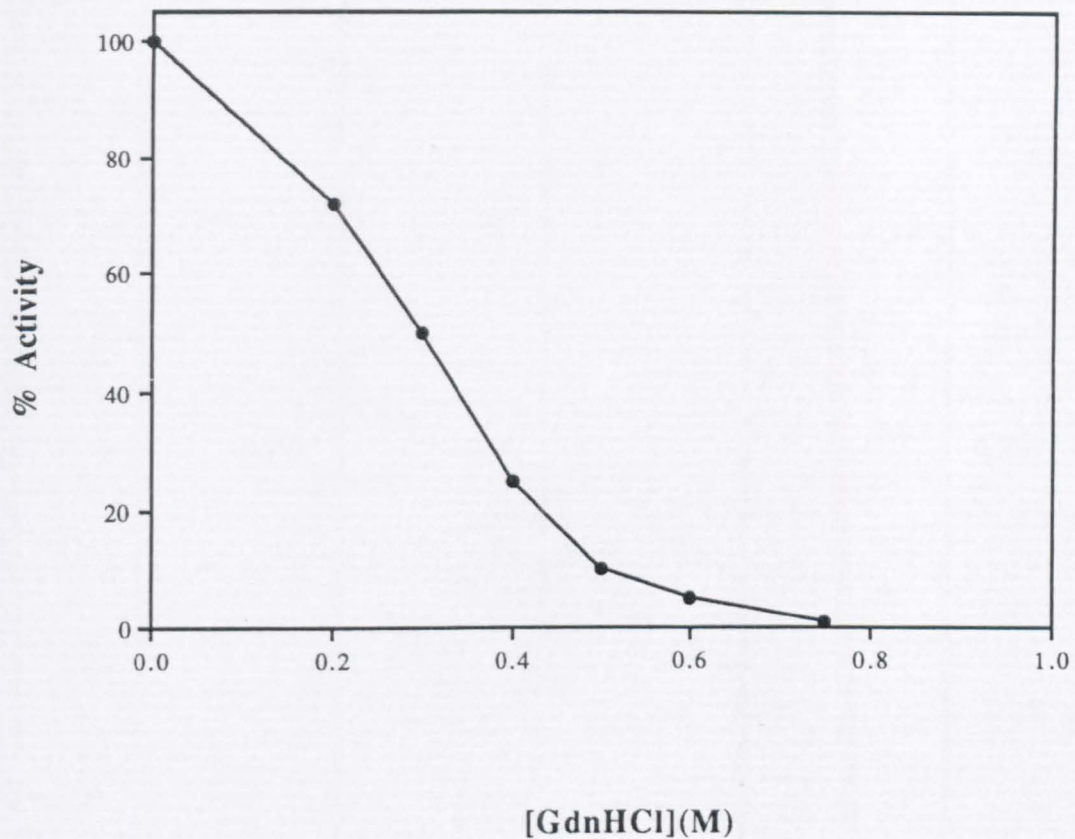


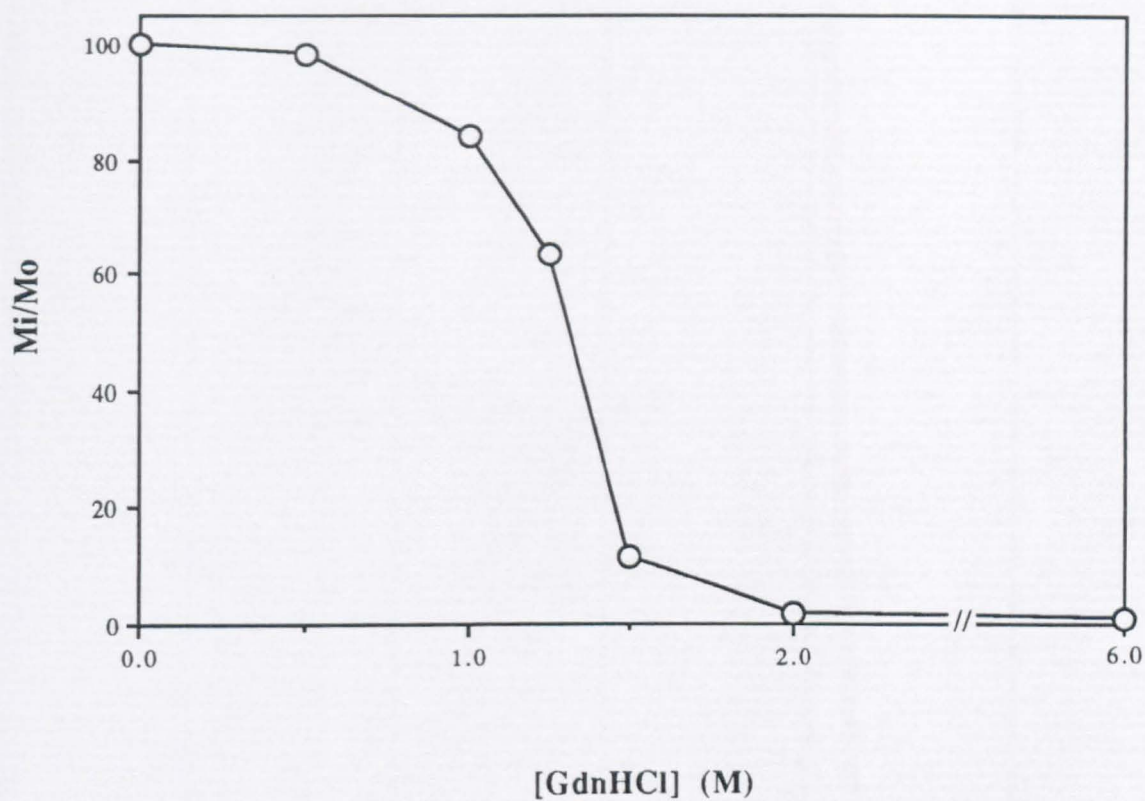
Figure 7.1 The far u.v. c.d.spectrum of GroEL (0.25mg/ml)

The spectrum was recorded at 20 °C in 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate and 0.1mM dithiothreitol (pH7.5)



**Figure 7.2** Loss of ATPase activity of GroEL in the presence of GdnHCl

Data are expressed relative to a control sample of GroEL which was recorded in the absence of denaturant. The concentration of GroEL was 40 $\mu$ g/ml and the buffer was 50mM potassium phosphate, 20mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate, containing 0.1mM dithiothreitol (pH 7.5) at 20°C.



**Figure 7.3** Changes in the  $M_r$  of GroEL in the presence of GdnHCl as determined by light scattering at 360nm

The concentration of GroEL was 50 $\mu$ g/ml in 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate, containing 0.1mM dithiothreitol (pH 7.5) at 20°C.

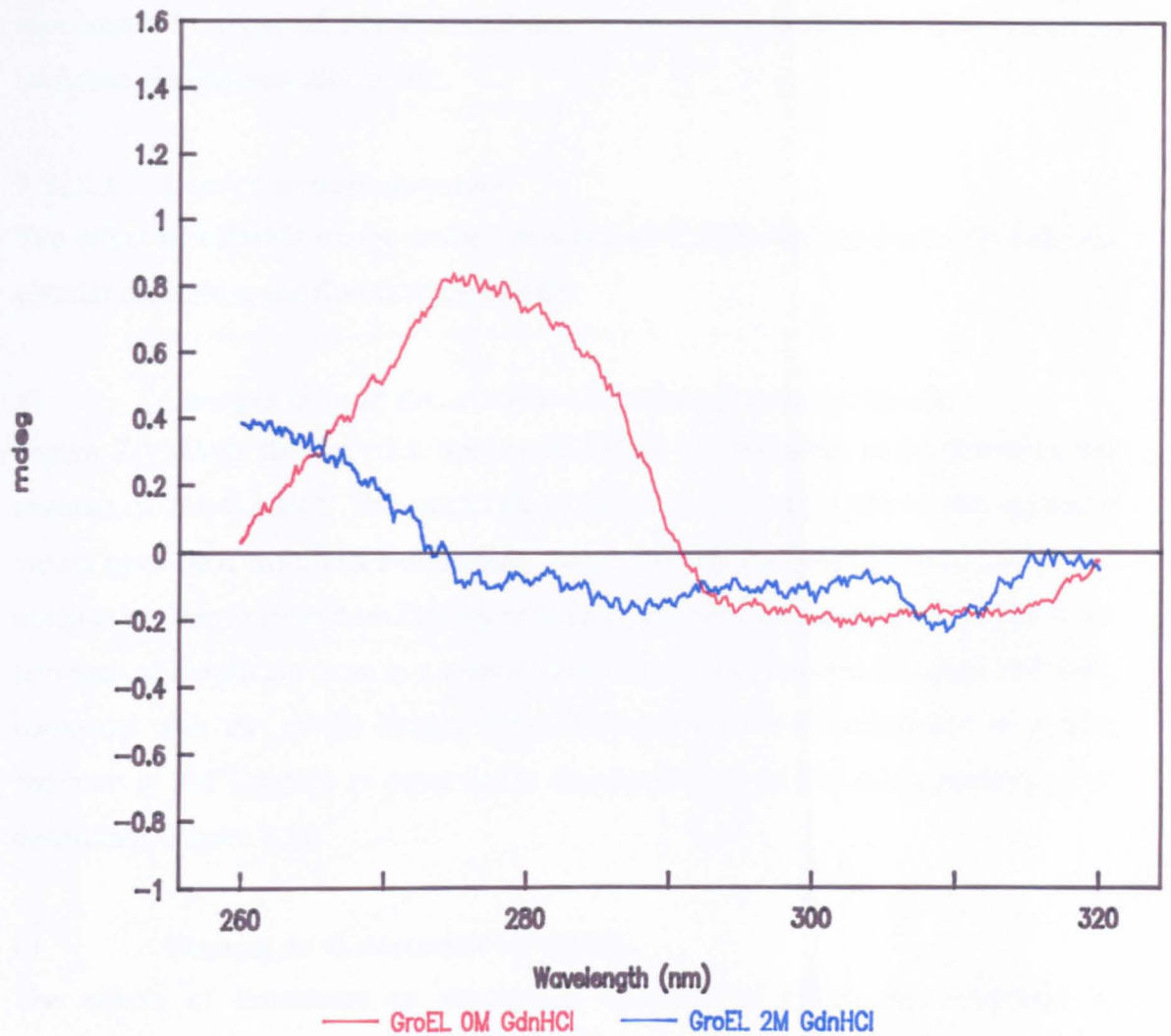


Figure 7.4 The near u.v. c.d. spectra of GroEL (0.75mg/ml) in the presence and absence of 2M GdnHCl

Spectra were recorded at 20 °C in 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate and 0.1mM dithiothreitol (pH 7.5)

light scattering) as a function of GdnHCl concentration. It can be observed that as the concentration of denaturant is increased beyond about 1M there is a substantial loss of quaternary structure. At approximately 2M GdnHCl the GroEL is dissociated into monomers. Lissin *et al.* (1990) found that in urea the conversion of monomers was complete at a concentration of 4M.

### **7.3.2.3 Loss of tertiary structure**

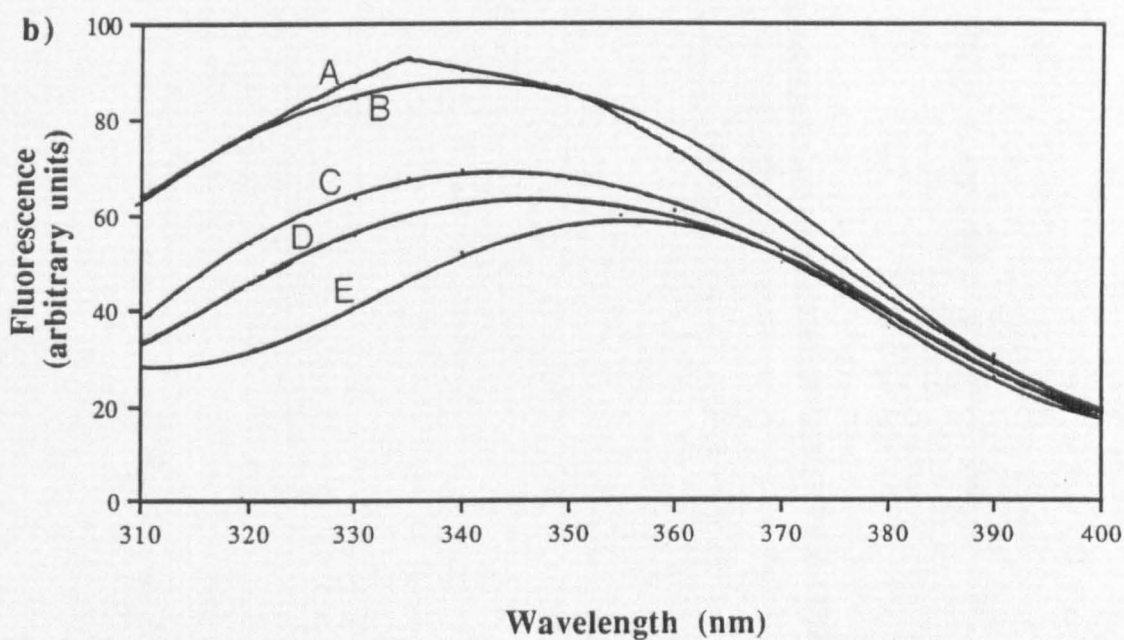
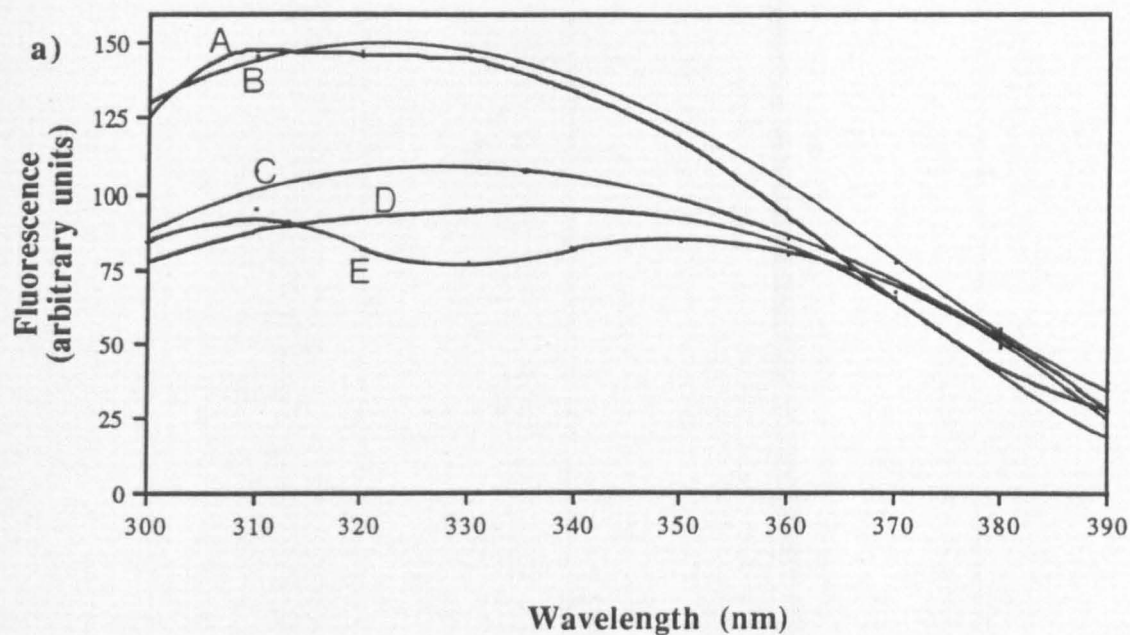
The effect of GdnHCl on the tertiary structure of GroEL was monitored by near u.v. circular dichroism, and fluorescence studies.

#### **a) Changes in near u.v. circular dichroism spectra of GroEL**

Figure 7.4 shows the near u.v. spectra of GroEL (0.75mg/ml) in the presence and absence of 2M GdnHCl. The amplitude of signal is relatively weak in this region. It would have been worthwhile examining more concentrated preparations but this was not possible due to limited availability of GroEL. In essence it can be gleaned that in the presence of denaturant there is a marked reduction in the intensity of signal of GroEL compared with that of the control. This presumably reflects the collapse of tertiary structure in 2M GdnHCl as noted in the fluorescence studies at this concentration of denaturant (Figure 7.5).

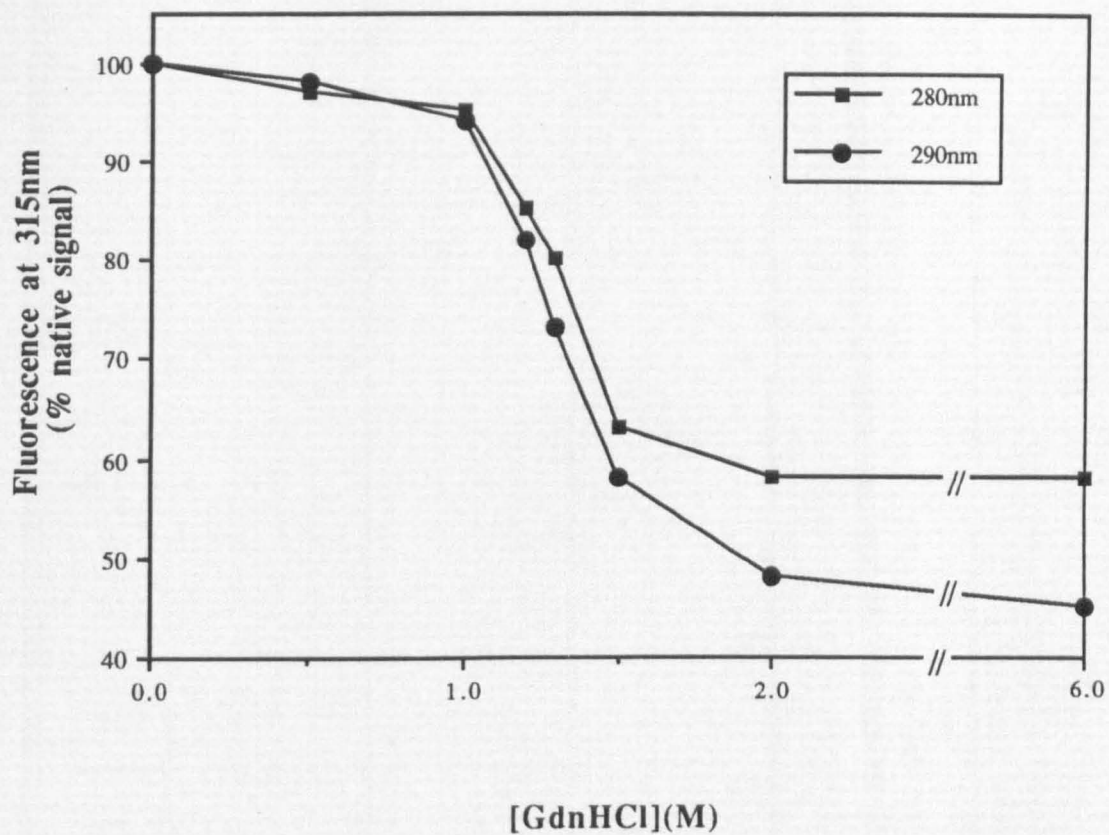
#### **b) Changes in fluorescence of GroEL**

The effects of denaturant on the tertiary structure of GroEL as monitored by fluorescence studies are shown in Figures 7.5a and 7.5b. The spectra in Figure 7.5a (excitation 280nm) show the quenching of the intrinsic fluorescence of GroEL as the concentration of denaturant increases. In the absence of denaturant the spectrum can be seen to have a rather broad peak. The wavelength of maximum emission occurs between 310nm and 335nm. This peak corresponds predominantly to the fluorescence of tyrosine side chains which have been reported to have an emission wavelength of 305nm (Brand and Witholt, 1967) although shifts in emission can be effected by environmental factors, such as solvent and/or neighbouring amino acyl residues. When the GdnHCl concentration exceeds approximately 1.5M there is a shift in the emission maximum towards the red. At 6M GdnHCl the emission maximum is shifted to approximately 350nm which is close to the characteristic wavelength at which fully solvated tryptophan residues fluoresce. Excitation at 280nm allows the contribution of



**Figure 7.5** Changes in the fluorescence of GroEL in the presence of GdnHCl

Figures 7.5 (a) and 7.5 (b) represent excitation at 280nm and 290nm respectively. Spectra of GroEL (50 μg/ml) were recorded at 20°C in 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate, containing 0.1mM dithiothreitol (pH 7.5). Spectra A, B, C, D and E refer to GdnHCl concentrations of 0, 1.0, 1.3, 1.5 and 6.0M respectively.



**Figure 7.5c** Changes in the fluorescence at 315nm of GroEL in the presence of GdnHCl

Data were recorded using the conditions described for Figures 7.5a and 7.5b.

both tyrosine and tryptophan residues to be measured. It was decided to examine the contribution of tryptophan predominantly by exciting at 290nm. Figure 7.5b shows a similar pattern of unfolding to Figure 7.5a in that the intensity of fluorescence decreases and shifts to the red as the concentration of GdnHCl is increased. In Figure 7.5c the changes in intensity at 315nm of GroEL are expressed as a function of GdnHCl.

It is noteworthy that the DNA derived sequence of GroEL indicates that the chaperonin is devoid of tryptophan residues (Hemmingsen *et al.*, 1988). In contrast, the fluorescence studies presented in this chapter and previous work of Price *et al.* (1991) clearly indicate the presence of tryptophan in the GroEL preparation. Given the “promiscuous” binding nature of GroEL it is possible that tryptophan-containing proteins and peptides may co-purify with the chaperonin and thus give rise to misleading results. The presence of contaminating proteins, which co-purify with GroEL and are not detectable by Coomassie staining, has been reported by Hayer-Hartl and Hartl (1993). Alternatively, the overexpression of GroEL in plasmids with a high copy number could lead to mutations in the sequence of the chaperonin. The implications of this possibility could be that either a mutation was present in the DNA from which the original sequence data was obtained or that the tryptophan-containing protein obtained from the plasmids used by Price *et al.* (1991) was the product of a mutation which may have occurred during overexpression. Santos and Tuite (1993) have discussed, in a recent review, the possible errors which can occur during the translation of proteins in overexpression systems. In their review they highlight the problems of mistranslation due to frameshifting and the misincorporation of amino acids into protein sequences due to limited availability of the required tRNA. In the case of bacteriophage T4 it has been noted that the availability of Trp-tRNAs is important to ensure that frameshifting during translation is kept to a minimum. Sequence analysis of both the original gene and plasmid products would be necessary to verify or refute the existence of such errors in translation.

#### **7.3.2.4 Loss of secondary structure**

The c.d. spectra of GroEL incubated at 20 °C and 2 °C in the absence and presence of increasing concentrations of GdnHCl are shown in Figures 7.6a and 7.6b respectively. Figure 7.6c shows the change in ellipticity at 225nm as a function of GdnHCl



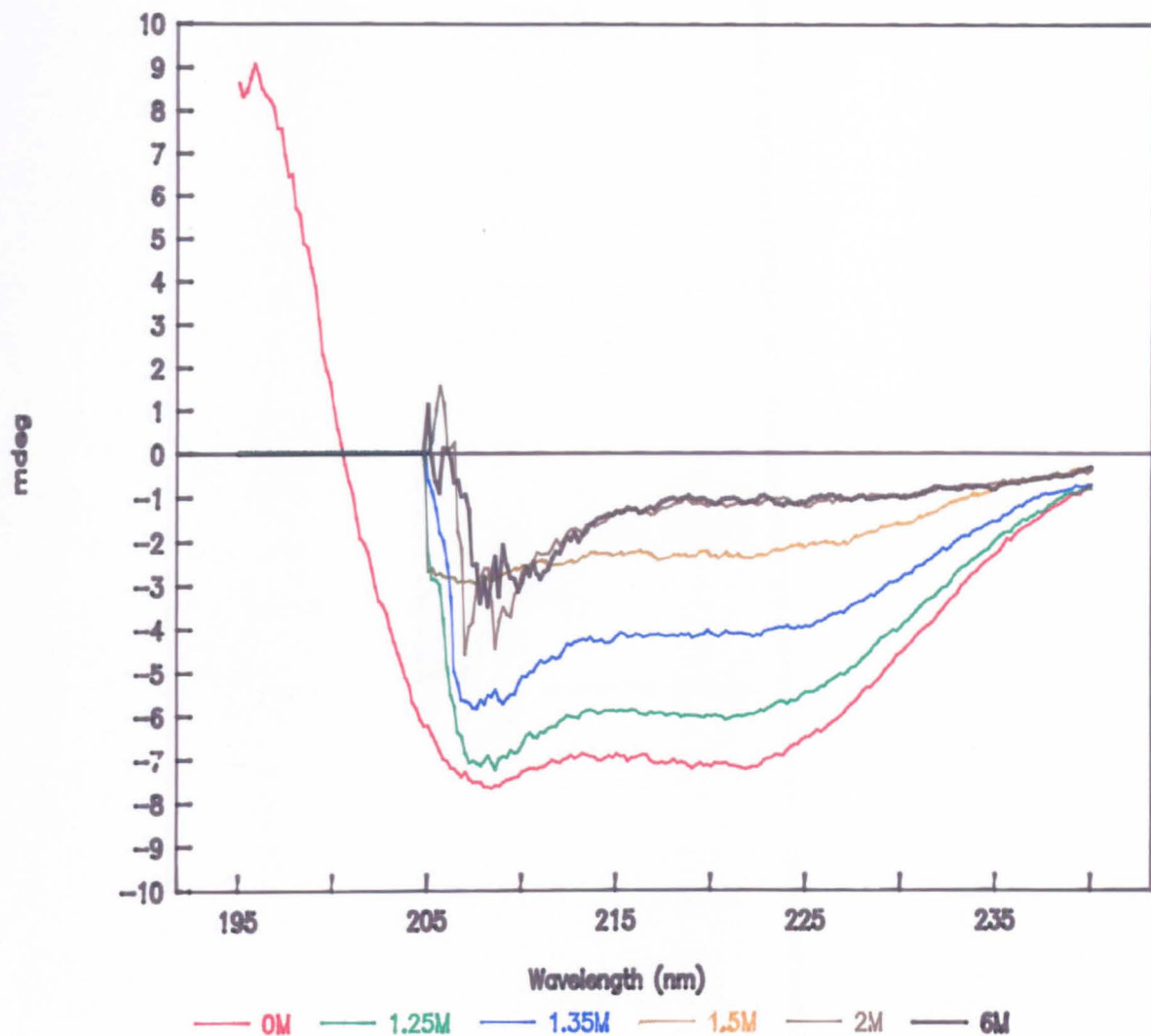


Figure 7.6a The c.d. spectra of GroEL (50 $\mu$ g/ml) incubated at 20 °C in the presence and absence of increasing concentrations of GdnHCl

Spectra were recorded in 50mM potassium phosphate, 20mM potassium acetate and 0.1mM dithiothreitol (pH 7.5) containing the indicated GdnHCl.

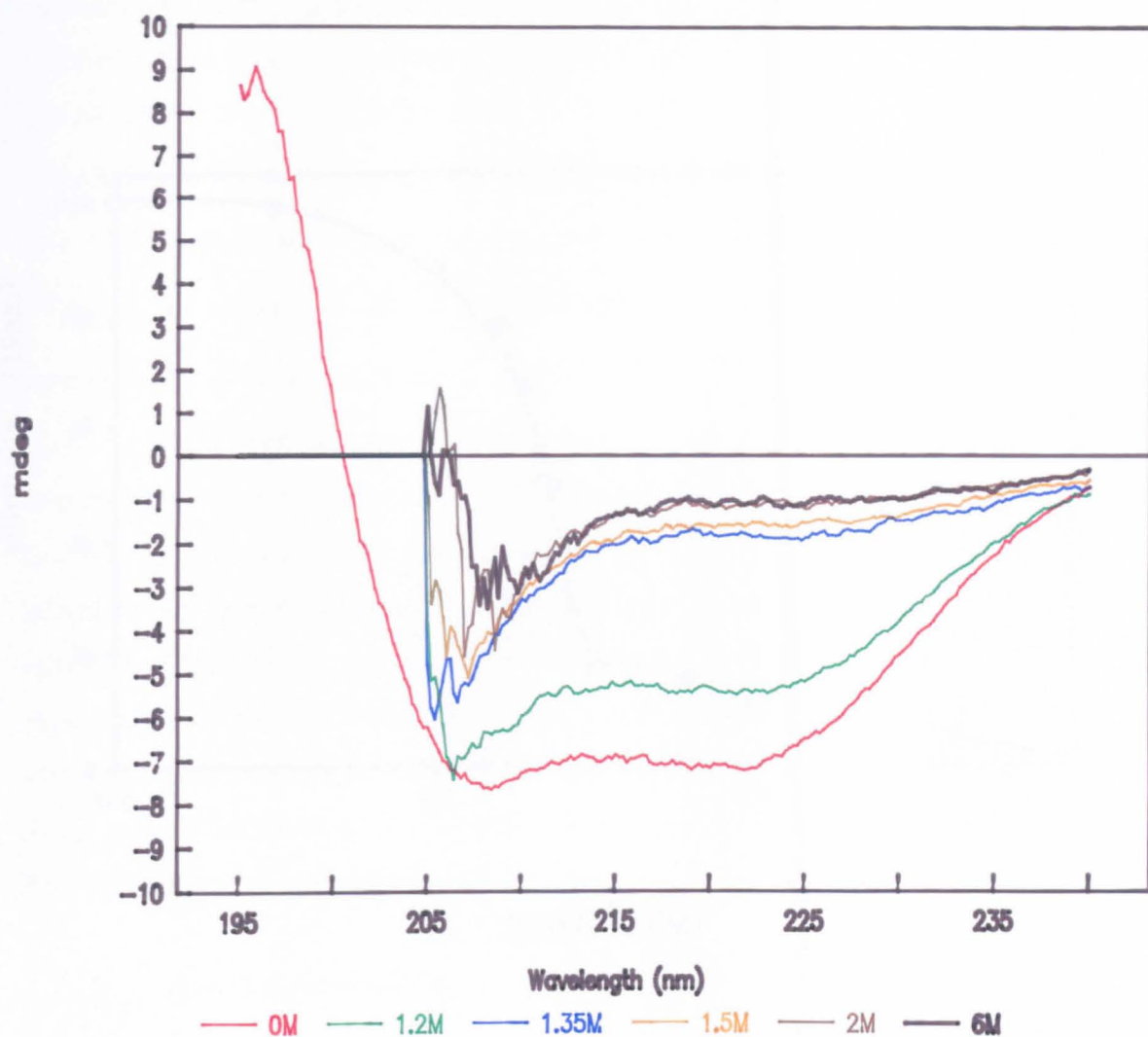
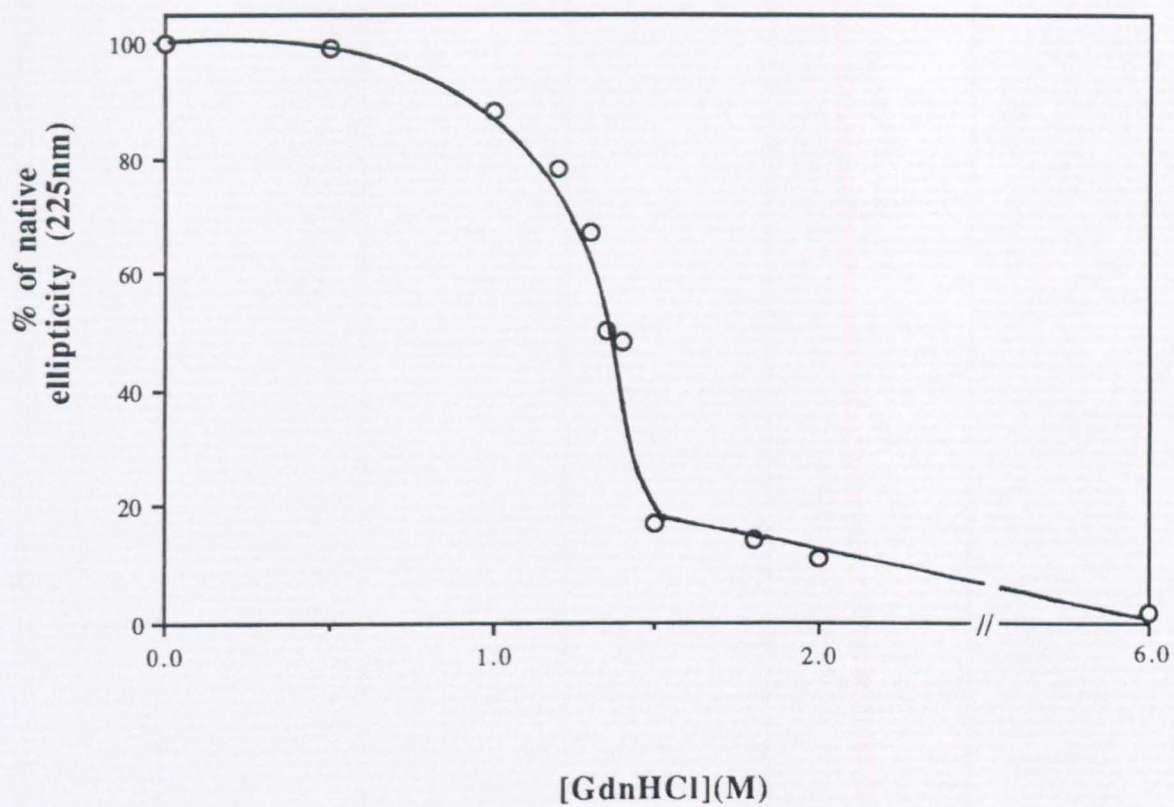


Figure 7.6b The c.d. spectra of GroEL (50μg/ml) incubate at 2 °C in the presence and absence of increasing concentrations of GdnHCl

Spectra were recorded using the conditions described in Figure 7.6a.



**Figure 7.6c** Changes in the ellipticity at 225nm of GroEL as a function of GdnHCl concentration

Data were recorded using the conditions described for Figures 7.6a and 7.6b. (20°C).

concentration. In separate experiments the effects of incubating GroEL with urea at 20 °C and 2 °C was also examined and the results are shown in Figures 7.7a and 7.7b, respectively. There is a marked change in the secondary structure of GroEL in GdnHCl (concentrations in the range from 1 to 1.5M) and the mid-point of unfolding occurs at approximately 1.35M GdnHCl (Figure 7.6a). GroEL is less stable in the presence of GdnHCl at 2 °C with the mid-point of unfolding occurring at approximately 1.25M GdnHCl (Figure 7.6b). The pattern of unfolding observed in the presence of urea was found to be similar. At 20 °C the mid-point of unfolding is reached in a urea concentration of 3.3M while at 2 °C the mid-point occurs at approximately 3.1M. Lissin *et al.* (1990) observed similar results in their work on the disassembly and reassembly of GroEL using urea as the denaturant. In this work they noted that the decrease in temperature assists the disassembly of the GroEL oligomer in the presence of denaturant. It was suggested that since hydrophobic interactions are reduced at low temperatures, it follows that such interactions must be important in maintaining the oligomeric structure of GroEL. In all cases described above the unfolding of GroEL was found to be much more cooperative than other oligomers notably fumarase (section 3.3.2) and citrate synthase (section 5.3.2) suggesting, perhaps, a very high degree of stabilisation of each subunit within the oligomer.

### **7.3.3 Attempted refolding of GroEL**

#### **7.3.3.1 Reactivation of ATPase**

Following incubation in various concentrations of GdnHCl and subsequent dialysis to remove the denaturant, the activity of ATPase was measured. The results are summarised in Figure 7.8. It was found that the success of reactivation was influenced by the concentration of GdnHCl in the initial incubation mix. At an initial concentration of 1M GdnHCl or below (where only slight changes in structure occur) the activity of ATPase could be restored following the removal of denaturant by dialysis. The efficiency of reactivation was found to diminish as the concentration of GdnHCl in the initial incubation mix exceeded 1M. At concentrations of GdnHCl greater than 2M relatively little ATPase activity was restored following dialysis.

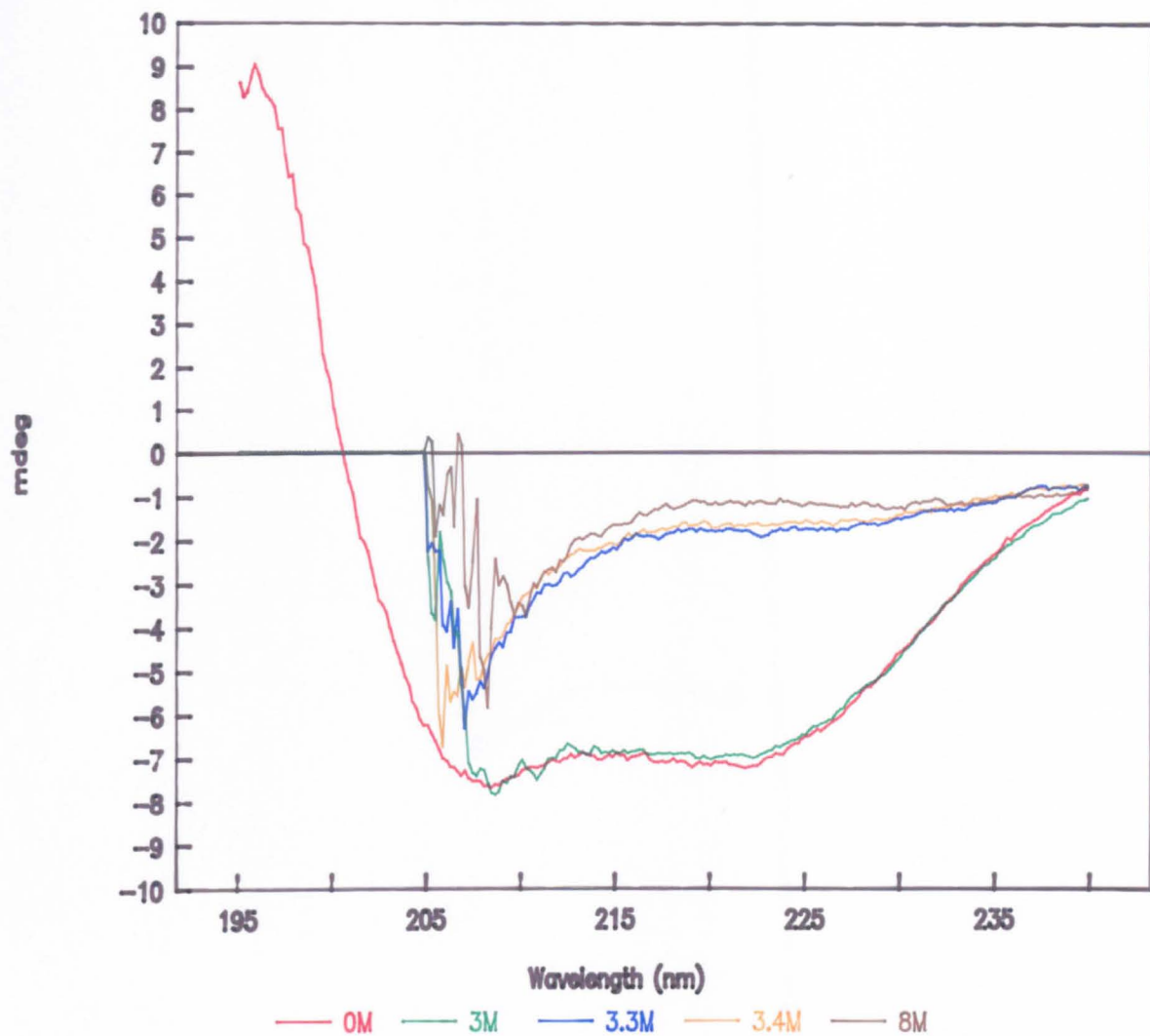


Figure 7.7a The c.d. spectra of GroEL (50 μg/ml) incubated at 20 °C in the presence and absence of increasing concentrations of urea

Spectra were recorded in 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate, 0.1mM dithiothreitol (pH 7.5) containing the indicated concentration of urea.

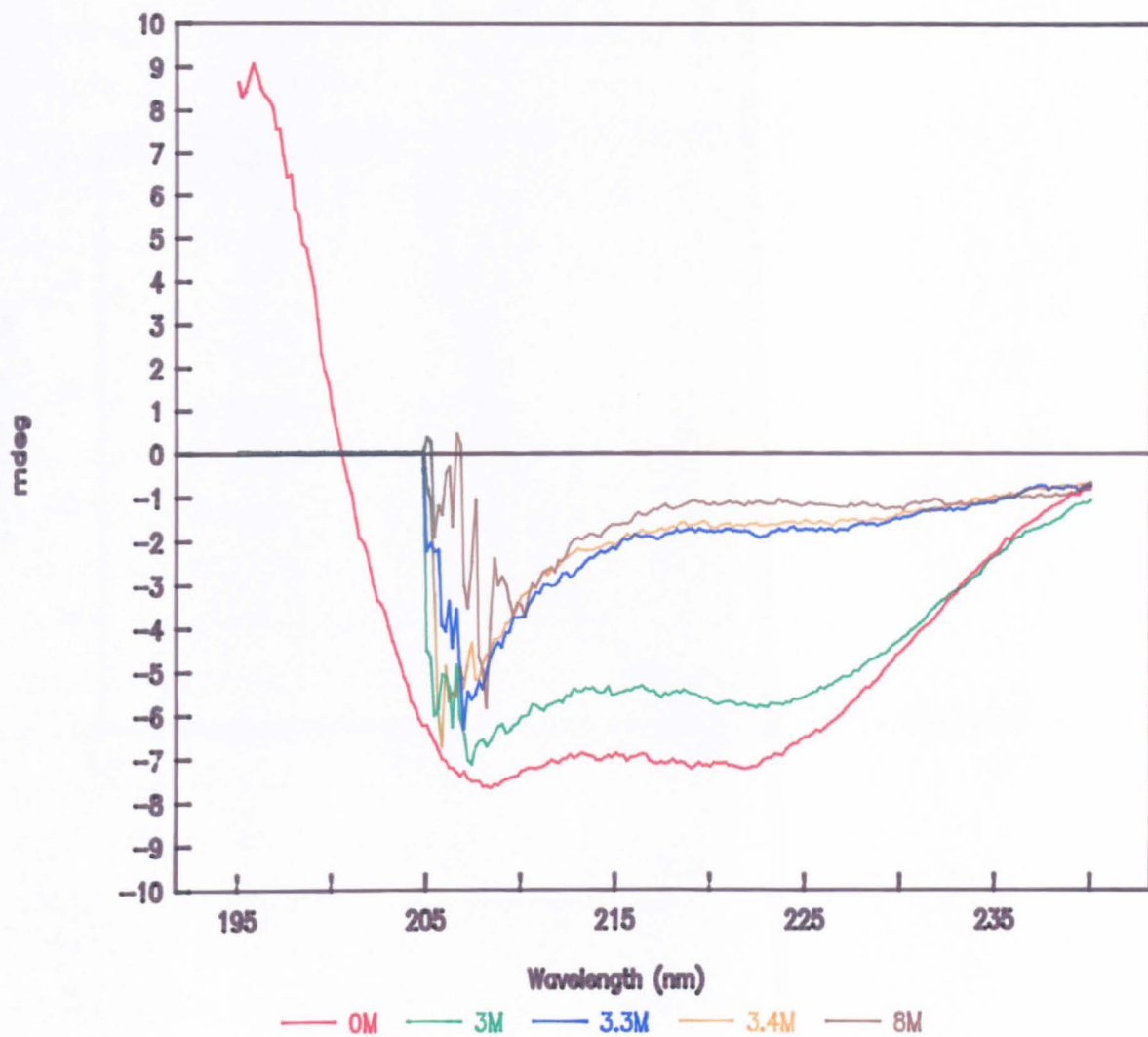
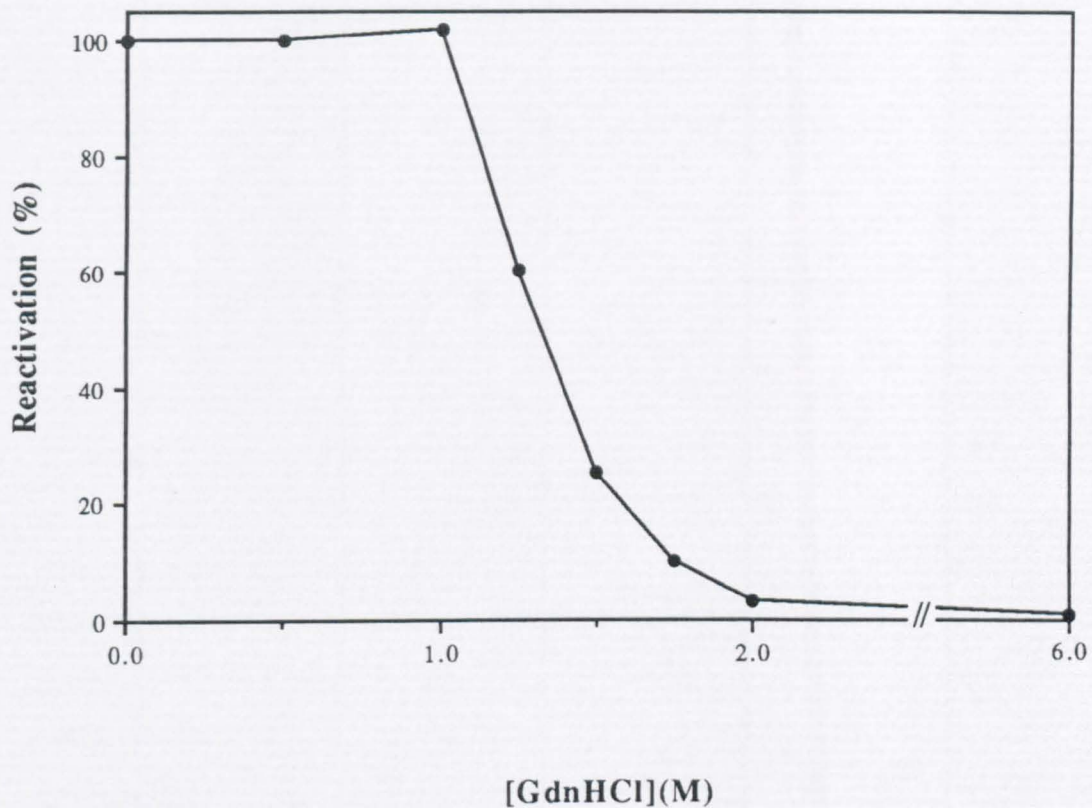


Figure 7.7b The c.d. spectra of GroEL (50 $\mu$ g/ml) incubated at 2 °C in the presence and absence of increasing concentrations of urea

Spectra were recorded using the conditions described in Figure 7.7a.



**Figure 7.8** The reactivation of GroEL after incubation in GdnHCl and subsequent dialysis

The ATPase activity of GroEL (50 $\mu$ g/ml) was recorded in 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate, containing 0.1mM dithiothreitol (pH 7.5).

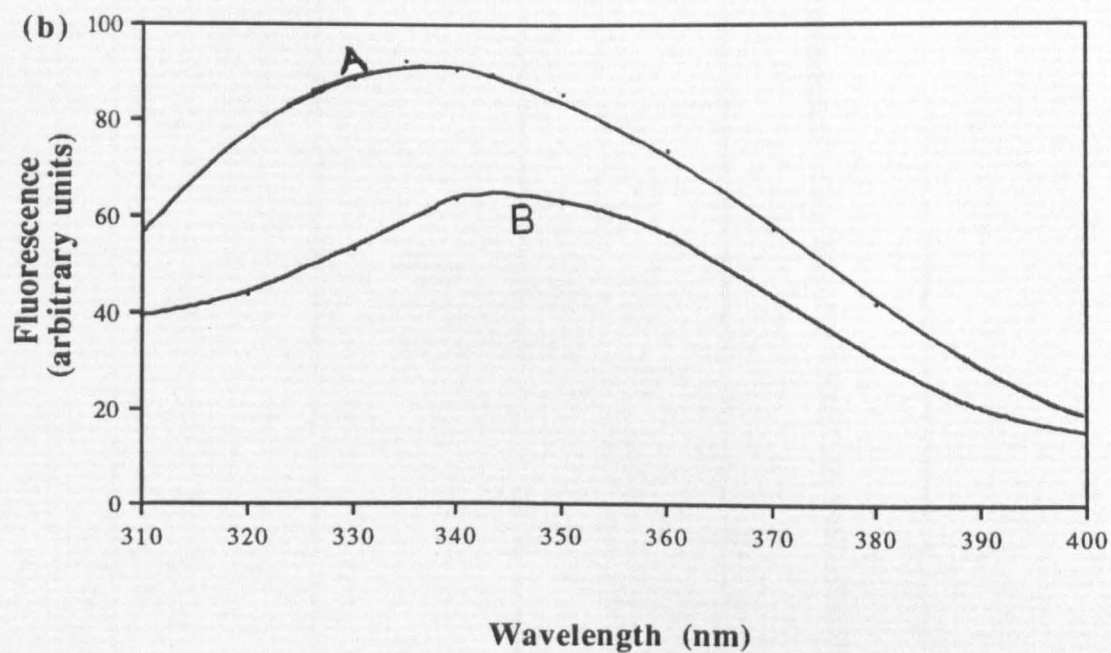
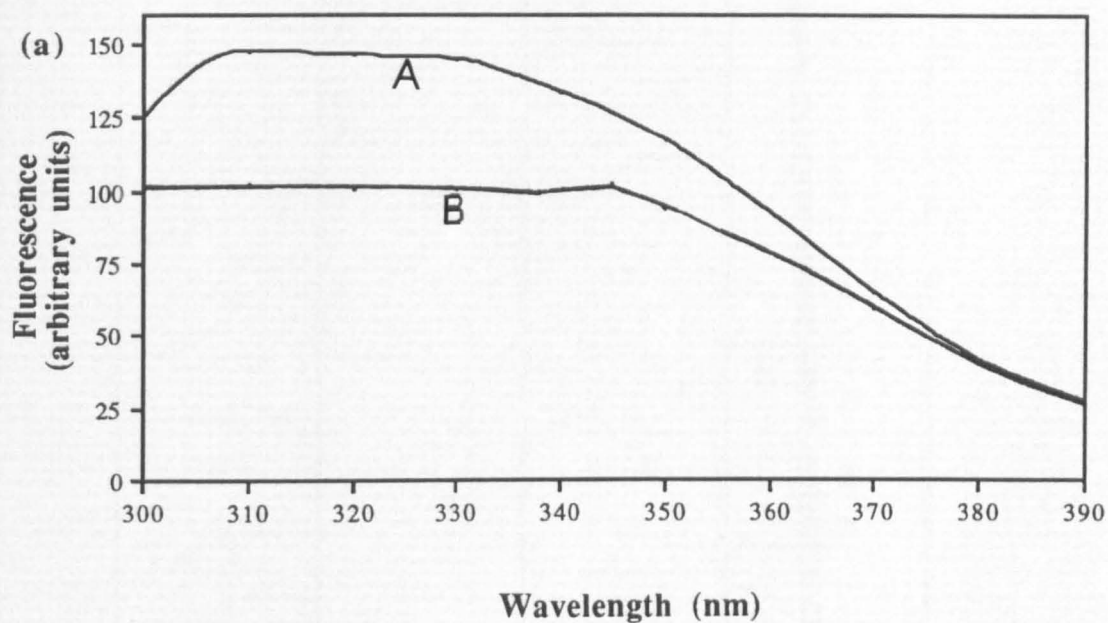
### **7.3.3.2 Regain of light scattering properties**

At initial concentrations of GdnHCl of 1M and below (at which point only slight changes occur) the light scattering properties of GroEL following dialysis were found to be essentially the same as the native oligomer indicating the regain of quaternary structure. When the initial concentration was above 1M GdnHCl it was found that the intensity of light scattering was about 50% less than the control sample which had been incubated in the absence of denaturant and subsequently dialysed. The formation of aggregates was also noted in some cases following incubation at high concentrations of GdnHCl.

### **7.3.3.3 Regain of tertiary and secondary structure**

The regain of tertiary and secondary structure as monitored by fluorescence and circular dichroism are shown in Figures 7.9 (a & b) and 7.10, respectively. It was found that the extent of regain of these structural elements following dialysis was also dependent on the initial concentration of GdnHCl in the incubation mix. At initial GdnHCl concentrations of 1M and below, GroEL was able to refold with maximal efficiency relative to the control protein which was incubated in the absence of denaturant and subsequently dialysed. Fluorescence measurements of samples incubated in concentrations of GdnHCl greater than 1M similarly indicated that the regain of tertiary structure following dialysis was incomplete. Indeed, Figures 7.9a and 7.9b show that following complete denaturation in 6M GdnHCl and subsequent dialysis, the intensity of fluorescence at 335nm is only about 65% of the control and the wavelength maximum occurs at 345nm compared with the maxima of native and unfolded GroEL (335nm and 355nm respectively). At initial concentrations greater than 1M although the amplitude of the c.d. signal at 225nm was at least 90% of that of the control sample (Figure 7.10), the c.d. minimum usually observed under native conditions at 208nm, was shifted to a slightly lower wavelength. In their studies on the disassembly and reassembly of GroEL Lissin *et al* (1990) observed that the c.d. spectrum of monomeric GroEL displayed a similar shift to a slightly lower wavelength. It can also be seen from these results that when the chaperonin was dialysed at 2 °C the extent of regain of secondary structure was reduced by at least 30%.





**Figure 7.9** Fluorescence spectra of GroEL (50 $\mu$ g/ml) following dialysis: (a) and (b) represent excitation at 280nm and 290nm respectively

Spectra A and B refer to samples incubated in the absence and presence of 6M GdnHCl and then dialysed against 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate 0.1mM, containing dithiothreitol pH 7.5 at 20°C.

#### **7.3.3.4 ANS binding studies**

It has been suggested by Pritsyn *et al.* (1990) that the molten globule state may represent a general type of intermediate in protein folding (section 1.6.4.1). Given the observation that the regain of secondary structure of GroEL was essentially complete following unfolding and subsequent dialysis whereas the regain of higher structural elements was less efficient, it was decided to examine each of the denatured/dialysed samples of GroEL for the presence of the molten globule state. There was a small 2.2-fold enhancement in the fluorescence of ANS (20 $\mu$ M) in the presence of native GroEL (50 $\mu$ g/ml) relative to free ANS. In the presence of increasing concentrations of GdnHCl the enhancement was reduced by up to 30% relative to the native chaperonin. Following the removal of the denaturant from the GroEL samples there was only a 2-fold enhancement in the fluorescence of ANS compared with free ANS. Thus by this criterion it could be concluded that the folding intermediate obtained following dialysis is not in a typically molten globular state.

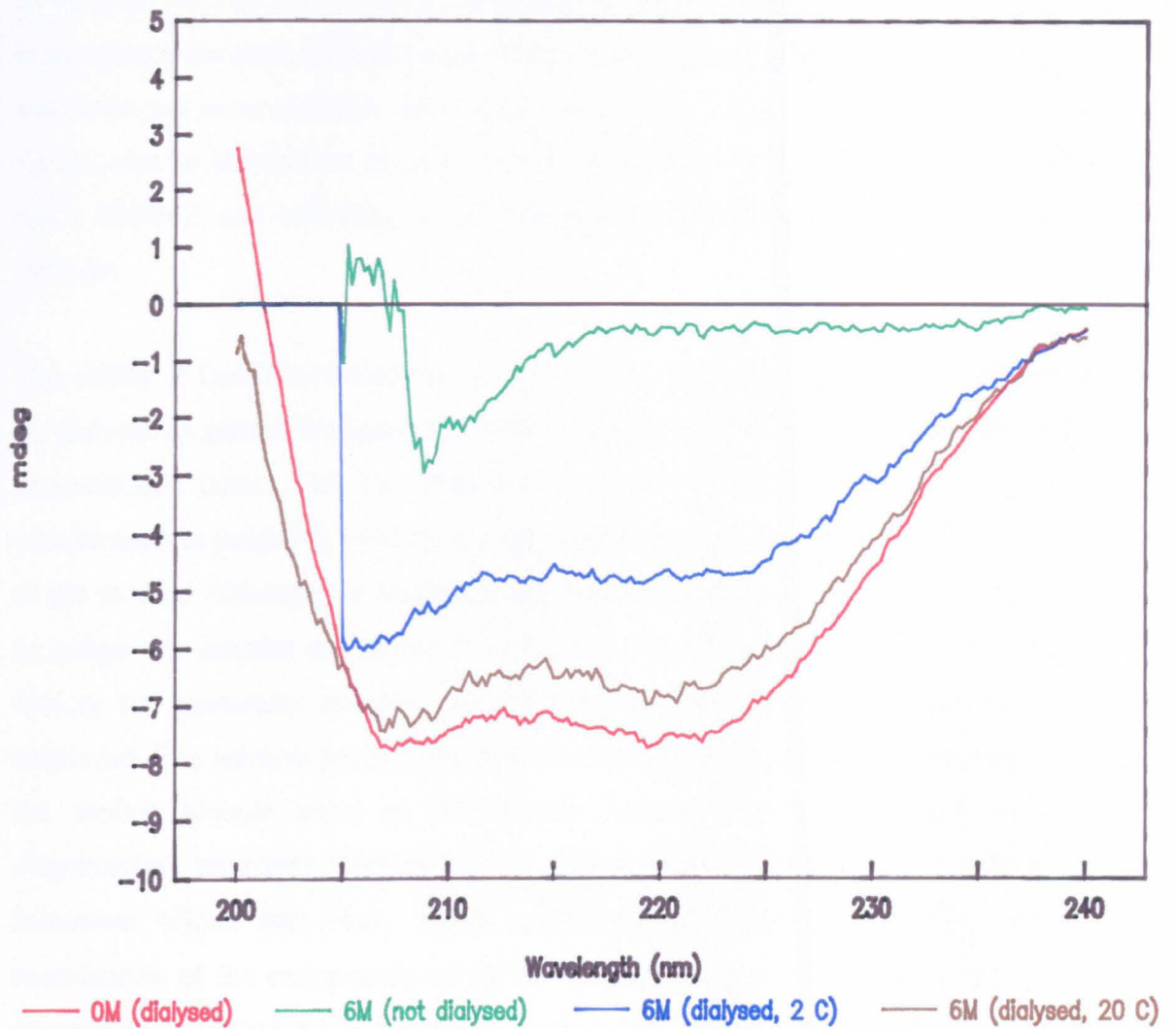


Figure 7.10 The far u.v. c.d. spectra of GroEL (50 $\mu$ g/ml) following incubation in GdnHCl and subsequent dialysis at 20 °C or 2 °C

Spectra were recorded at 20 °C in 50mM potassium phosphate, 20mM potassium acetate, 5mM magnesium acetate, 0.1mM dithiothreitol (pH 7.5). The spectra of native (0M dialysed) and denatured (6M not dialysed) enzyme have been included for reference

## 7.4 Summary of Results

The  $\alpha$ -helical content of the bacterial chaperonin, GroEL, has been estimated to be in the range from 44% to 58% using the CONTIN procedure. The ATPase activity of GroEL is sensitive to the perturbations caused at the active site under mild denaturing conditions which do not seem to affect the overall conformation of the protein. Disassembly of GroEL can be effected by relatively low concentrations (1M-1.5M) of the chaotropic agent GdnHCl and unfolding of the tetradecamer proceeds in a highly cooperative manner.

The ability of GroEL to refold has been examined following the removal of denaturant by dialysis. In general it appears that following incubation in GdnHCl beyond a critical concentration (about 1M) the chaperonin is unable to reassemble into a functional tetradecamer as judged by catalytic activity and the examination of the structural features of the protein. Although the secondary structure of GroEL can be effectively regained, as judged by circular dichroism, it is evident from fluorescence studies that native tertiary and quaternary structure are not achieved under the experimental conditions employed. The inactive product obtained on refolding does not show characteristics of the molten globule state, an intermediate feature of several proteins such as: dihydrofolate reductase (Garvey *et al.*, 1989); lysozyme (Martin *et al.*, 1991);  $\beta$ -lactamase (Goto and Fink, 1989);  $\alpha$ -lactalbumin (Baumet *et al.*, 1989). Further examination of the end product on refolding was shown by Price *et al.* (1993) to be susceptible to proteolysis by thermolysin, unlike native GroEL. Using small amounts of proteinase a band was observed on SDS-PAGE which corresponded to a digestion product of  $M_r$  54 000. This product could be digested further on addition of more proteinase. From these results it was suggested that the inactive GroEL product obtained on attempted refolding is a folded structure of  $M_r$  54 000 which is overall much less compact than the native oligomer.

*Chapter eight*

**General Discussion**

## 8.1 General Conclusions and Discussion

### 8.1.1 Main objectives of thesis

This thesis outlines studies on a number of proteins which present particular problems as far as folding and assembly are concerned. All of the proteins examined consisted of more than one subunit and included: dimers (citrate synthases from pig heart and *Tp. acidophilum*); a tetramer (fumarase from pig heart); a hexamer (citrate synthase from *Ac. anitratum*); an octamer (NAD<sup>+</sup>-dependent isocitrate dehydrogenase); a 14mer (the bacterial chaperonin, GroEL). These oligomeric proteins were studied in an attempt to examine the intrinsic ability of such proteins to refold, following denaturation, in view of the side reactions which compete with the association of subunits during the folding process. In these studies particular elements of the protein structure during unfolding and following refolding were characterised. From these data a measure of the stability of the proteins towards denaturation was provided. Three of the proteins examined are localised in the mitochondrial matrix *in vivo* and are synthesised as precursors in the cytosol before translocation. Previous work has shown that translocated proteins are difficult to refold *in vitro* (see for example: West and Price, 1988, 1990; West *et al.*, 1990). As mentioned in section 1.7 such proteins are generally in a non-native state prior to and during translocation which renders them vulnerable to proteases and non-productive interactions with other proteins which are present at high concentrations in the cellular milieu (Eilers and Schatz, 1986; Becker *et al.*, 1992). The folding and refolding of a thermophilic citrate synthase (dimer) was also investigated and compared with the results obtained for the mesophilic enzymes from gram negative bacteria (hexamer) and pig heart (dimer). Given the fact that high temperatures can compromise the stability of folding intermediates it was considered of interest to examine the ability of the thermophile to refold at both thermophilic (55 °C) and mesophilic temperatures (20 °C).

### 8.1.2 Estimation of secondary structure by circular dichroism

Estimations of the secondary structure content of each protein was obtained by application of the CONTIN procedure of Provencher and Glöckner (1981). From this group of proteins reliable X-ray structural data has only been obtained for the citrate synthases from pig heart (Remington *et al.*, 1982) and *Tp acidophilum* (Russel, PhD thesis, Russel *et al.*, 1993). Crystals have been obtained from GroEL (Svensson *et al.*, 1994) however, the X-ray data for this protein have not yet provided an indication of its secondary structural content. It will be interesting to see whether the CONTIN secondary structure analyses are consistent with future X-ray crystallographic data. In the case of citrate synthase from pig heart, which has been shown by X-ray crystallographic data to contain 72% helix, the values obtained using far u.v. circular dichroism data indicated a somewhat (of the order of between 12 and 20%) lower figure for the  $\alpha$ -helical content. As noted in section 5.3.1 this may reflect a limitation in the ability of the CONTIN secondary structure analysis program to distinguish between  $\beta$ -sheet structure and the  $\beta$ -sheet-like properties of the 8  $\alpha$ -helical "sandwich" at the dimer interface of this enzyme (Remington *et al.*, 1982). It is noteworthy that the recent crystallographic data obtained for *Tp. acidophilum* citrate synthase (with which the pig heart enzyme c.d. spectrum is almost superimposable) indicate an  $\alpha$ -helical content of 62%. On the basis of the circular dichroism results obtained (section 6.3.1) these workers recalculated the content of  $\alpha$ -helix in the pig enzyme using the coordinates reported by Remington *et al.* (1982) and the data analysis program used to interpret the coordinates of the *Tp. acidophilum* enzyme; the results from this analysis suggest that the pig heart enzyme contains 60%  $\alpha$ -helix. The similarity between these citrate synthases is striking despite the low (20%) sequence identity between them and presumably reflects the redundancy of the "folding code" (Jaenicke, 1987).

### 8.1.3 Sensitivity of the active site toward GdnHCl

All of the proteins, with the exception of the bacterial citrate synthases, were found to lose catalytic activity in the presence of low concentrations ( $\leq 0.5\text{M}$ ) of GdnHCl prior to any major structural changes. This finding presumably reflects the sensitivity of the active sites of these enzymes towards perturbations caused by the presence of GdnHCl. A similar effect of GdnHCl (at low concentrations) on the active site has been noted for other enzymes, notably: glyceraldehyde-3-phosphate dehydrogenase (Tsou *et al.*, 1983);

creatine kinase (Yao *et al.*, 1985; Tsou, 1986); ribonuclease (Lui and Tsou, 1987). This behaviour could be attributed to structural perturbations at the active site which render the enzyme inactive or it could indicate that the GdnHCl itself is interacting with residues at the active site of the protein thus, acting as an inhibitor of enzymatic activity (Creighton, 1990). Although the guanidinium cation is the effective unfolding agent (Mayr and Schmidt, 1993) at low concentrations GdnHCl acts as a source of anions which can bind to the positively charged groups of amino acid side chains (Hagihara *et al.*, 1993). It has been proposed that the active sites of some enzymes are situated in molecular regions which are more flexible than the molecule as a whole (Tsou, 1986). This proposal is consistent with the finding that the active sites of several enzymes are located at a flexible interface between two domains or subunits and involve the binding of substrate or co-factor to each domain or subunit. For example citrate synthase undergoes a conformational change upon binding oxaloacetate and acetyl-CoA and the active site of this enzyme is composed of amino acids from both subunits (Remington *et al.*, 1982). Zhang *et al.* (1993) used thermal denaturation of adenylate kinase to show that, in accord with the results obtained using GdnHCl, inactivation occurred before any noticeable conformational changes thus lending support to the proposal of Tsou (1986) that the active site is more sensitive to denaturing conditions than is the rest of the molecule. By contrast the citrate synthases from both *Tp. acidophilum* and *Ac. anitratum* are seemingly activated at concentrations of GdnHCl  $\leq 0.4\text{M}$  and  $\leq 0.7\text{M}$ , respectively. Succinimide quenching studies indicate that the latter citrate synthases are more rigid than the pig heart enzyme in the absence of denaturant. Although it is difficult to know whether “local” or “general” rigidity is important, it may well be the case that the active sites of the *Tp. acidophilum*. and *Ac. anitratum* enzymes are located in a less flexible region than their pig heart counterpart and therefore the “loosening” effect of the denaturant could lead to conformational flexibility important for the efficiency of the catalytic cycle.

#### 8.1.4 Unfolding studies

In the case of the unfolding of fumarase and citrate synthase (from pig heart) the changes in tertiary structure occur slightly ahead of the changes in secondary structure following incubation in GdnHCl. This pattern of unfolding has also been observed for other proteins, for example: glyceraldehyde-3-phosphate dehydrogenase (Xie and Tsou,



1987); rhodanase (Tandon and Horowitz, 1989); papain (Xaio *et al.*, 1993); adenylate kinase (Zhang *et al.*, 1993). In isocitrate dehydrogenase the changes in secondary structure occur slightly ahead of the changes in tertiary structure. The pattern of unfolding observed for GroEL suggests that it is a highly cooperative process with the changes in quaternary, tertiary and secondary structures running in parallel. It is envisaged that once the interactions which stabilise the association between the subunits of the tetradecamer are disrupted the free energy increases and the folded conformation becomes unstable; this results in the complete unfolding of the oligomer over quite a narrow range (between 1.0M and 1.5M) of GdnHCl concentrations. The differential stabilities of the three citrate synthase were examined towards both chemical and thermal denaturation. At higher concentrations of denaturant (greater than 1.5M) it appears that the thermophilic enzyme is least stable towards GdnHCl denaturation while, the hexameric enzyme is most stable in GdnHCl compared with the other citrate synthases. It is probable that this increased stability arises from the interactions between the six subunits but this will require structural characterisation of the hexameric enzyme. In thermal studies it was shown that, as expected, the thermophilic enzyme is more stable towards thermal denaturation than the citrate synthase from *Ac. anitratum* which, in turn, is more stable than the pig heart enzyme. Comparisons of the structures of other thermophilic and mesophilic proteins have shown that the extra stability of thermophiles is provided by an increase in the number of non-covalent interactions which stabilise all proteins. These additional interactions serve to make the protein more rigid than their mesophilic counterpart (Varley and Pain, 1991; Jaenicke, 1991). The results obtained from the study of the thermal and chemical denaturation of citrate synthase from *Tp. acidophilum* suggest that the covalent interactions by which the stabilisation towards temperature is afforded by this enzyme are not sufficient to stabilise the enzyme towards the perturbations effected by GdnHCl. It is probable that the unfolding pathway of the enzyme is different under the two methods of denaturation.

#### **8.1.5 Refolding studies**

Studies on the refolding and reactivation of the proteins examined indicate that the recovery of functional protein following denaturation, at high ( $\geq 4M$ ) GdnHCl concentrations, is generally inefficient although optimising the refolding conditions can lead to significant levels of recovery. Only the dimeric citrate synthases can be

reactivated to a moderate extent (approximately 55% when refolding conditions are optimised) using the dilution procedure to initiate refolding. The results obtained using the dialysis procedure suggest that this method of initiating the refolding of the proteins examined is generally more successful although GroEL and the hexameric citrate synthase could not be refolded correctly by any of these methods. Following dialysis the recoveries of activity for the proteins which could be refolded were: fumarase, 48%; NAD<sup>+</sup>-dependent isocitrate dehydrogenase, 75%, pig heart and thermophilic citrate synthases 55-60%. The success of the dialysis procedure over the dilution procedure is presumably due to the gradual lowering of the concentration of GdnHCl allowing the protein to make correct structural adjustments between intermediates on the folding pathway. It is possible that low concentrations of GdnHCl may have a stabilising effect on these intermediates at certain stages in the folding pathway. As mentioned previously (section 5.3.2.3) Hagihara *et al.* (1993) have recently reported that concentrations of GdnHCl, up to 1M, are able to bring about the refolding of apomyoglobin and cytochrome c (following acid-induced unfolding) by stabilising the molten globule state. The successful refolding of the proteins examined is dependent on other factors such as the initial concentration of protein in the incubation mix, the presence of dithiothreitol, temperature and the buffer system used in the dilution or dialysis steps.

The citrate synthases are extremely sensitive to changes in the refolding conditions and the yield of active enzyme decreases markedly as the protein concentration increases above about 20µg/ml. Aggregation appears to be a competing factor in the folding pathway at higher protein concentrations which explains the low recovery of soluble material under these conditions (Jaenicke, 1987). Interestingly the refolding of the thermophilic citrate synthase was found to be less successful (≤10% reactivation) when dialysis or dilution of the denaturant was performed at the optimal growth temperature (55 °C) of this organism. This may be due to the instability of transient intermediates in the unfolding pathway or may indicate that a non-native conformation is favoured at the higher temperature. In the case of NAD<sup>+</sup>-dependent isocitrate dehydrogenase reactivation appears to be optimal at higher (about 50µg/ml) protein concentrations and declines significantly when the protein concentration is reduced (≤ 30µg/ml); the recoveries of secondary and tertiary structure are essentially the same at both concentrations. Given that this enzyme is an octamer it is probable that the interactions

between subunits are diminished at the lower protein concentrations as reflected in the lower regain of catalytic activity. The inability of the GroEL protein to reassemble into a functional oligomer following denaturation in GdnHCl and subsequent dialysis has confirmed the findings of Lissin *et al.* (1990). These workers have proposed that the *in vitro* reassembly of GroEL following denaturation in urea proceeds by way of a “self chaperoning” mechanism involving GroES and the hydrolysis of ATP. *In vivo* studies of the assembly of mitochondrial chaperonin hsp60 using the defective yeast mutant, *mif4* (Cheng *et al.*, 1990) have indicated the need for the functional pre-existing hsp60 oligomer for assembly to occur. It remains to be established whether GroEL directs the correct assembly of the GroEL complex by way of a “self chaperoning” mechanism *in vivo* or whether other factors or chaperones are involved in this process. The characterisation of the folding intermediate(s) will be of use in elucidating the structure/function role of GroEL.

## 8.2 Protein folding *in vivo*

It is clear from these studies that certain oligomeric proteins are difficult to refold *in vitro* and that other factors can influence the success of the refolding process. The way in which such factors assist in this process *in vitro* raises some interesting questions regarding the situation *in vivo*. For example: what prevents aggregation of the folding intermediates of proteins in a cellular milieu where the protein concentration is extremely high; similarly, how do nascent precursor polypeptides which are translocated in an unfolded or non-native conformation avoid non-productive interactions between their exposed hydrophobic regions and those of neighbouring polypeptides? In recent years the quest to find an answer to such questions has intensified considerably with the discovery that “helper” proteins, known collectively as chaperones, are involved in mediating protein folding *in vivo*. Chapters 1 and 7 have introduced the concept of chaperone proteins, with the latter concentrating more specifically on the chaperonins GroEL and GroES. The characterisation and studies on the mechanism of action of these “helper proteins” are paving the way to a fresh understanding of the protein folding process in the cell. Recent evidence has emerged which questions the proposed cycle of events illustrated in chapter 7 (Diagram 1). At least three groups (Azem *et al.*, 1994; Schmidt *et al.*, 1994; Todd *et al.*, 1994) have reported the existence (in addition to the asymmetric complex) of a symmetric GroEL-GroES heterooligomer in which one

GroES is bound to each end of a single GroEL oligomer thus obstructing the openings to the central cavity. This symmetric complex which resembles the shape of a rugby ball can assist the ATP-dependent refolding of the Rubisco enzyme (Azem *et al.*, 1994). Schmidt *et al.* (1994) have illustrated the symmetrical conformation of this complex using electron microscopy and image analyses. Furthermore Todd *et al.* (1994) have proposed an alternative dynamic model to that proposed by Martin *et al.* (1993; see also section 7.1). The newly proposed model suggests the involvement of the symmetric heterooligomer in the cycle of interactions between GroEL, GroES, non-native protein and MgATP.

The involvement of an increasing number of other chaperone proteins in mediating protein folding in the cell has been reported (Table 8.1 -for review see: Gething and Sambrook, 1992). Recent experiments suggest that several different molecular chaperones act in concert to assist the folding of nascent polypeptide chains both co-translationally and following release from the ribosome machinery. *In vitro* studies (Frydman *et al.*, 1994) using rabbit reticulocytes and incorporating messenger RNA for the enzyme luciferase have highlighted the formation of a complex between the ribosomal-bound nascent chain and three chaperone proteins, namely, hsp70, hsp40 and TCP-1 chaperonin (which has been suggested to be the cytosolic equivalent to the hsp60 chaperonin family, Ellis, 1990). The results obtained by these workers suggest that hsp70 and hsp40 bind to the nascent polypeptide chain during biosynthesis to prevent mis-folding of the chain; the “protected” chain is subsequently passed to the TCP-1 containing chaperone and when the polypeptide is released from the ribosome the chaperonin assists in the correct folding of the protein (Frydman *et al.*, 1994).

In conclusion it is evident from the *in vitro* studies presented in this thesis that the autonomous refolding of certain proteins without the manipulation of experimental conditions and the presence of additional factors often leads to the formation of non-functional, non-native products. Aggregation is a major side reaction in the folding of these proteins *in vitro* and subunit association, being of a higher than first order process, is prone to following this non-productive, competing pathway at elevated protein concentrations. It appears that the efficiency of the folding of proteins *in vivo* is both assisted by enzymes which catalyse slow folding steps and mediated by chaperone

**Table 8.1†** Chaperone proteins and their known functions in the cell

Subcellular location	Organism	Component	Subunit $M_r$ (kDa)	Known functions
<b>The hsp70 family</b>				
Cytosol	<i>E. coli</i>	Dnak	69	Co-operation with the heat shock proteins DnaJ and GrpE in $\lambda$ DNA replication; activation of RepA for binding to PI DNA; dissociation of protein aggregates.
	Yeast	Ssa1-4p	69-72	stabilization of precursor proteins for translocation into ER and mitochondria; binding to nascent polypeptides.
	Mammals	hsc70		Uncoating of clathrin-coated vesicles
Mitochondria	Yeast	Ssc1p	70	Membrane translocation, folding of imported proteins
Endoplasmic reticulum	Yeast	Kar2p	78	protein translocation into the ER; assembly within the ER;
		BiP, Grp78	70	retention of misfolded proteins
<b>The hsp60 family</b>				
Cytosol	<i>E. coli</i>	GroEL	58	Folding of monomeric and multi-subunit proteins
		GroES	10	
Mitochondrial matrix	Fungi, mammals	hsp60, hsp58	58-64	Folding and assembly of newly imported proteins
		hsp10	10	
Chloroplasts	Plants	Rubisco subunit binding protein	61 ( $\alpha$ -chain) 60 ( $\beta$ -chain)	Folding and assembly of rubisco and probably of other proteins imported into chloroplasts
SecB	<i>E. coli</i>		17	Stabilization of precursor proteins for export across the cytoplasmic membrane
<b>The hsp90 family</b>				
Cytosol	Mammals	hsp90	90	Stabilization of steroid hormone receptors in a conformation competent to bind hormone; association with pp60 <sup>src</sup> before its phosphorylation; facilitation of refolding by preventing aggregation

†Taken from Hlodan and Hartl (1994) In Mechanisms of Protein Folding (Ed. R. H. PAIN) Table 1 p202. IRL Press at Oxford University Press, Oxford

proteins which have thus far been shown to protect nascent polypeptides from non-productive interactions in the cell. The mechanisms by which these helper proteins assist in the folding process remain to be established although their characterisation is well underway. A number of problems which have yet to be resolved include:

(i) the mode of association of the chaperone proteins with target proteins; (ii) the stoichiometry of this association; (iii) the specificity of association (bearing in mind the alleged ability of GroEL to bind to a variety of target proteins, Viitanen *et al.*, 1992) and, (iv) the role of the central cavity in the action of chaperones upon proteins (particularly multi-subunit proteins); for instance if the folding process involves processes which occur in the central cavity are subunits dealt with individually prior to assembly?

### 8.3 Future work

In the light of the results obtained in this thesis, future work will focus on characterising the possible role of chaperones in the refolding of each of the proteins examined. The inability of the *Tp acidophilum* citrate synthase to refold at 55 °C compared with the significant reactivation at 20 °C raises some interesting questions regarding the mechanism by which proteins from this and other thermophiles attain their native conformation *in vivo* at optimal growth temperatures. Given the instability of folding intermediates at elevated temperatures it would be interesting to investigate the way in which the protein folding process in thermophiles and indeed, other extremophiles can proceed under extreme conditions.

During the course of the early part of the work carried out on citrate synthase (chapter 5) the ability of chaperonins to suppress the aggregation of pig heart citrate synthase was reported by Buchner and co-workers (1991). It is interesting to note that the efficiency of refolding of this enzyme using chaperone proteins was not dissimilar to the values obtained in chapter 5 using optimised *in vitro* conditions. The development of the latter may be of use in the successful recovery of functional proteins from "inclusion bodies".

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## ***Appendix***

## Appendix 1 Secondary structure estimates from circular dichroism

The method of Provencher and Glöckner (1981) uses the CONTIN program to fit the observed spectrum to combinations of the “best fit” reference spectra of  $\alpha$ -helices,  $\beta$ -sheets and other elements of structure in proteins (rather than in model polypeptides). It uses a reference set of 16 well-characterised proteins. Overall we would put most confidence in this method, provided that good data are obtained down to 190nm and that the estimate of protein concentration is reliable. According to Provencher (personal communication) it is not reliable to try to obtain estimates of  $\beta$ -turn within the “remainder” portion.

The method of Chang *et al.* (1978) uses a smaller reference set of standard proteins. In the “constrained” method the sums of the various types of structure add up to 100%; in the “free” no such limitation is placed on the values. The results obtained sometimes contain negative values for particular structural features; mathematically this may be reasonable but clearly biochemically it is not.

The method of Siegel *et al.* (1980) uses data over a narrow range of wavelengths (210 to 240 nm) to estimate  $\alpha$ -helix content. It can be useful if the data are only reliable over a restricted range of wavelengths, due to for instance, high absorbance.

One point to note is that Chen *et al.*, (1974) report that the mean residue weight c.d. signal due to an  $\alpha$ -helix is actually a function of the  $\alpha$ -helix length, increasing to a limit as the  $\alpha$ -helix lengthens. Thus for proteins with very high  $\alpha$ -helix contents or with very long stretches of  $\alpha$ -helix, the CONTIN reference spectra may not be entirely appropriate and may overestimate the %  $\alpha$ -helix. This could be the case in non globular or membrane-spanning proteins for instance.

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# The unfolding and attempted refolding of citrate synthase from pig heart

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Key words: Citrate synthase; Protein unfolding; Protein folding; Circular dichroism; Mitochondrial protein assembly

The unfolding of the dimeric enzyme citrate synthase from pig heart in solutions of guanidinium chloride (GdnHCl) was studied. Data from fluorescence, circular dichroism (CD) and thiol group reactivity studies indicated that the enzyme was almost completely unfolded at GdnHCl concentrations  $\geq 4$  M. On dilution of GdnHCl, essentially no reactivation of the enzyme occurred. The implications of this finding for the process of folding and assembly in vivo of this and other mitochondrial enzymes are discussed. Exposure of the enzyme to high pH (9-10) led to only a small loss of secondary structure and partial reactivation could be observed on readjustment of the pH to 8.0.

## Introduction

Citrate synthase (citrate oxaloacetate-lyase((pro-3S)- $\text{CH}_2\text{COO}^- \rightarrow$  acetyl-CoA), EC 4.1.3.7) catalyses the condensation of acetyl-CoA and oxaloacetate to form citrate; this reaction is unique among those of the tricarboxylic acid cycle in that a new carbon-carbon bond is formed. Under certain conditions the reaction can be the rate-limiting step in the operation of the cycle [1] and theregulatory properties of citrate synthase from a variety of sources have been investigated [2]. The enzymes from animals, plants, fungi and archaebacteria are dimeric with a subunit  $M_r \approx 50\,000$ . By contrast, the enzymes from algae and Gram-negative bacteria consist of from 4 to 6 subunits (each of  $M_r \approx 50\,000$ ) [3]. The regulatory properties of the enzymes appear to be correlated with their quaternary structures [2].

Citrate synthase from pig heart has been particularly well studied with both the amino acid sequence [4] and X-ray structure [3,5] determined. The enzyme structure can be classified as essentially all  $\alpha$ -helix, with 20  $\alpha$ -helices (a number of which are kinked) in each subunit and only one short stretch of  $\beta$ -sheet. Each subunit consists of large and small domains containing 15 and 5 helices, respectively. The helices from the two subunits

pack together tightly to give a globular molecule; each active site contains significant contributions from amino acid side-chains of both subunits. On binding oxaloacetate or CoA, a marked conformational change occurs in which the small domain rotates relative to the large domain to give a 'closed' structure [3,5].

In eukaryotes, citrate synthase is coded for by nuclear DNA and synthesised in the cytosol as a precursor which is subsequently translocated into the mitochondria. The enzyme is thought to occur in the matrix [3], although there is some evidence that it may be bound to the inner mitochondrial membrane [6]. As part of our investigations of the assembly of translocated proteins [7-9] we have studied the unfolding and refolding of citrate synthase from pig heart. Our results show clearly that when the enzyme is extensively unfolded by GdnHCl, reactivation cannot occur on dilution of the denaturing agent. This situation has been found with other mitochondrial proteins, e.g., glutamate dehydrogenase [7] and aspartate aminotransferase [9]. It is likely that folding and assembly of such proteins in vivo may well involve the participation of special binding ('chaperone') proteins [10].

In earlier work [11], Wu and Yang had shown by hydrodynamic and CD measurements that in the presence of 6 M GdnHCl the pig heart enzyme dissociated into two disordered subunits. However, no attempt to reactivate the enzyme was reported. It has also been reported that the enzyme can be dissociated into monomers at pH 9.6 and that partial reactivation occurs on readjustment of the pH to 8.0 [12].

Abbreviations: GdnHCl, guanidinium chloride; Nbs<sub>2</sub>, 5,5'-dithiobis(2-nitrobenzoic acid); CD, circular dichroism.

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## Experimental procedures

Pig heart citrate synthase was purchased as an ammonium sulphate suspension from Sigma. The preparation was at least 95% homogeneous as judged by Coomassie blue staining following SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels [13]. By reference to the  $M_r$  values of marker proteins (Dalton Mark VII, Sigma) the subunit  $M_r$  was determined to be  $47\,000 \pm 2\,500$  compared with the  $M_r$  (48 969) calculated from the amino acid sequence [4]. The concentration of enzyme was determined spectrophotometrically at 280 nm, using  $A_{1\text{cm}}^{0.1\%} = 1.78$  [14].

Citrate synthase was assayed spectrophotometrically at 412 nm by the method of Srere et al. [15], in which the formation of CoA is determined by reaction with  $\text{Nbs}_2$ . Assay mixtures contained 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM oxaloacetate, 0.15 mM acetyl-CoA and 0.1 mM  $\text{Nbs}_2$ . The specific activity of the enzyme at 25°C was 120  $\mu\text{mol}/\text{min}$  per mg.

The unfolding and refolding of citrate synthase in GdnHCl was performed as described for analogous experiments with aspartate aminotransferase [8]. The enzyme was incubated in the appropriate concentration of GdnHCl in 20 mM Tris-HCl (pH 8.0) for 15 min at 0°C. Refolding was initiated by a 60-fold dilution of this sample into buffer at 20°C. Control experiments were performed in which GdnHCl was omitted from the initial incubation but included at the residual concentration in the buffer used for dilution. GdnHCl was AristaR grade (British Drug Houses); the concentrations of solutions of GdnHCl were checked by measurements of refractive index [16].

The number of reactive thiol groups on the enzyme (0.1 mg/ml) was determined by reaction with excess  $\text{Nbs}_2$  (250  $\mu\text{M}$ ) in 20 mM Tris-HCl (pH 8.0) at 20°C.

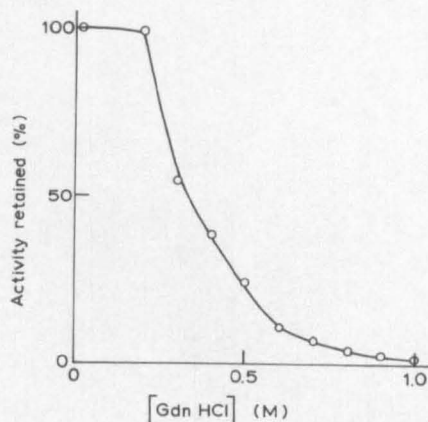


Fig. 1. Loss of activity of citrate synthase on incubation in GdnHCl. Enzyme (3.5  $\mu\text{g}/\text{ml}$ ) was incubated at 20°C for 15 min in 20 mM Tris-HCl (pH 8.0) and a sample was then taken for assay. Activities are expressed relative to a control sample from which GdnHCl was omitted.

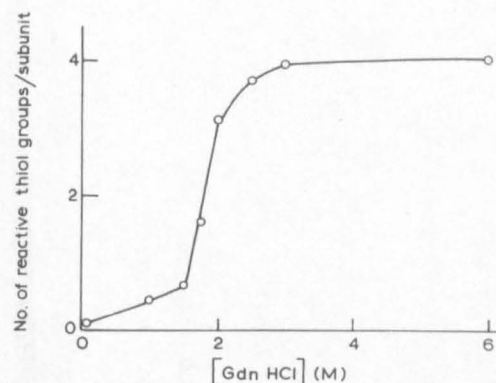


Fig. 2. The reactive thiol groups of citrate synthase. Enzyme (0.1 mg/ml) was incubated in 20 mM Tris-HCl (pH 8.0) at 20°C. The increase in  $A_{412}$  on reaction with  $\text{Nbs}_2$  (250  $\mu\text{M}$ ) was used to calculate the number of reactive thiol groups. In each case the reaction was complete within 10 min.

The reaction was complete within 10 min and the concentration of thiol groups calculated using the value of  $13\,600\text{ M}^{-1}\cdot\text{cm}^{-1}$  for the molar absorption coefficient at 412 nm of the thionitrophenolate ion [17].

Fluorescence measurements were made at 20°C in a Perkin-Elmer MPF 3A fluorimeter. Spectra were recorded 15 min after addition of GdnHCl to the enzyme. No further changes were observed after this time for at least 2 hours.

CD spectra were recorded at 20°C on a Jasco J-600 spectropolarimeter. Spectra in the near-UV (320–260 nm) and far UV (250–200 nm) were recorded in cells of pathlength 10 and 1 mm, respectively and at enzyme concentrations of 0.3 and 0.07 mg/ml, respectively. The observed ellipticity values were converted to mean residue ellipticity values using a value of 112 for the mean residue weight [4]. Spectra were recorded 15 min after addition of GdnHCl. No further changes were observed after this time for at least 2 h.

## Results

### Unfolding of citrate synthase in GdnHCl

**Activity measurements.** Enzyme (3.5  $\mu\text{g}/\text{ml}$ ) was incubated for 15 min at 20°C in 20 mM Tris-HCl (pH 8.0) in the presence of a given concentration of GdnHCl. A sample was then taken and assayed in the presence of the same concentration of GdnHCl. The results (Fig. 1) show that above 0.2 M there is a progressive loss of activity with increasing concentrations of GdnHCl, with only 10% activity remaining at 0.6 M.

**Reactive thiol groups.** In the absence of GdnHCl citrate synthase showed  $<0.1$  thiol groups per subunit reactive towards  $\text{Nbs}_2$ , in agreement with data reported by Srere [18]. In the presence of 6 M GdnHCl there were  $4 \pm 0.2$  reactive thiol groups per subunit, equal to the known number of cysteines in the polypeptide chain [4]. The extent of exposure of the thiol groups in the

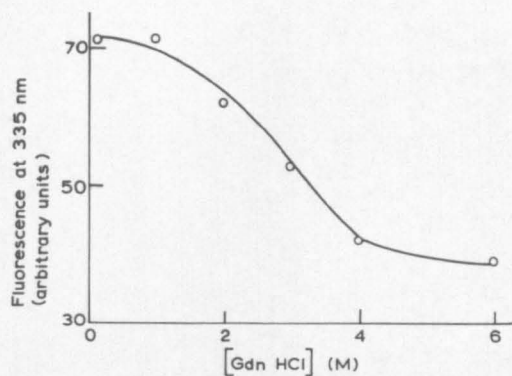


Fig. 3. Fluorescence of citrate synthase. Enzyme (16  $\mu\text{g}/\text{ml}$ ) was incubated in 20 mM Tris-HCl (pH 8.0) at 20°C. The excitation wavelength was 290 nm. The intensity of fluorescence at 335 nm is shown as a function of the concentration of GdnHCl.

presence of increasing concentrations of GdnHCl is shown in Fig. 2, where it is seen that the most marked changes occur in the range of GdnHCl concentrations from 1.5 M to 2.5 M.

**Fluorescence spectra.** When excited at 290 nm, citrate synthase showed a fluorescence emission maximum at 335 nm, characteristic of Trp side-chains partially shielded from the aqueous solvent. Addition of 1 M GdnHCl caused little change in the spectrum but higher concentrations of the denaturing agent caused a progressive quenching and shift in the emission maximum towards 350 nm, the value characteristic of fully exposed Trp side-chains. The fluorescence at 335 nm as a function of GdnHCl concentration is shown in Fig. 3.

**CD spectra.** The far-UV CD spectrum of citrate synthase (Fig. 4a) showed double minima at 222 nm and 210 nm, characteristic of a protein with significant  $\alpha$ -helical content [19]. Using the reference values for the mean residue ellipticity at these wavelengths [19], the %  $\alpha$ -helix can be estimated to be  $78 \pm 7\%$ , consistent with the value (72%) determined by X-ray crystallography [5]. The CD spectrum of native enzyme is in very good agreement with that reported by Wu and Yang [11]. However, their estimate of the helical content (50–55%) is rather lower than the present value because of their use of inappropriate reference values for the mean residue ellipticities of the various secondary structure features.

Fig. 4a shows the changes in the CD spectrum of the enzyme in the presence of increasing concentrations of GdnHCl. Up to concentrations of 1.5 M there is no significant change in the spectrum; however, above this concentration there is a progressive loss of secondary structure. The near-UV CD spectrum (Fig. 4b) also showed changes over this concentration range, reflecting the progressive disruption of the tertiary structure. From the data shown in Fig. 4c, it is clear that the changes in secondary and tertiary structure run broadly in parallel.

#### Attempted refolding of citrate synthase after denaturation

The ability of citrate synthase to regain activity after denaturation in GdnHCl was tested after 60-fold dilu-

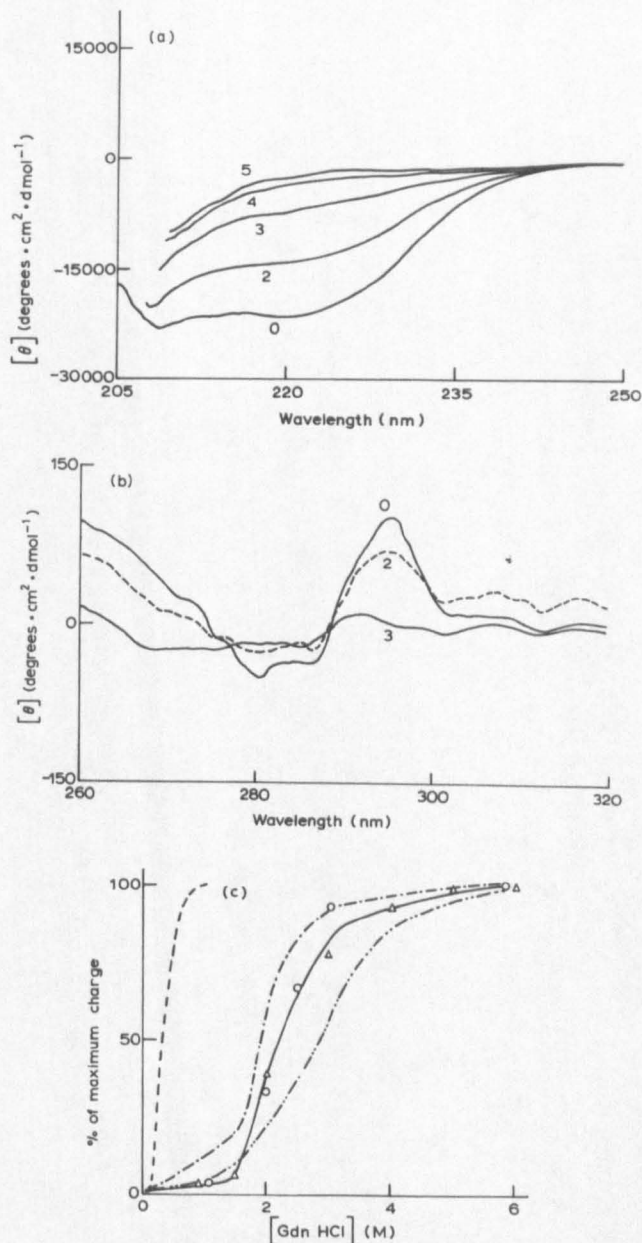


Fig. 4. CD of citrate synthase. Enzyme was incubated in 20 mM Tris-HCl (pH 8.0) at 20°C. (a) Far UV spectra. The enzyme concentration was 0.07 mg/ml; spectra were recorded in cells of pathlength 1 mm. The numbers by the traces indicate the molarity of GdnHCl when added. At higher concentrations of GdnHCl, the high absorbance below 210 nm leads to excessive noise. (b) Near-UV spectra. The enzyme concentration was 0.3 mg/ml; spectra were recorded in cells of pathlength 10 mm. The numbers by the traces indicate the molarity of GdnHCl when added. (c) Comparison of changes in the far-UV and near-UV ( $\Delta$ ), change in  $\theta_{225}$ ; ( $\circ$ ), change in  $\theta_{295}$ . In each case the change is expressed relative to the total change observed between native enzyme and a sample in the presence of 6 M GdnHCl. Data concerning changes in enzyme activity (-----), thiol group reactivity (-·-·-) and fluorescence (-·-·-) from Figs. 1, 2 and 3, respectively, are also shown.

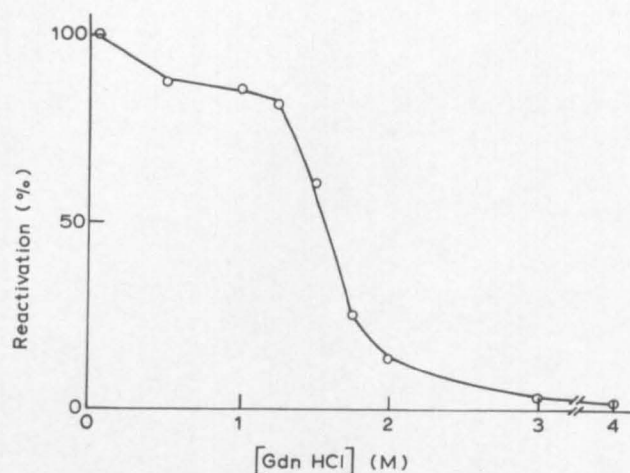


Fig. 5. Reactivation of citrate synthase after unfolding in GdnHCl. The unfolding and refolding of enzyme were performed as described in the text; the enzyme concentration during refolding was 4  $\mu\text{g}/\text{ml}$ . In each case activity is expressed relative to a control sample from which GdnHCl had been omitted in the initial incubation.

tion into 20-mM Tris-HCl (pH 8.0) as described in the Experimental section. As shown in fig. 5, nearly all ( $\geq 90\%$ ) activity could be regained if the initial GdnHCl concentration was  $\geq 1$  M. Above this concentration, the regain of activity declined sharply, reaching values of 15% and 3% at 2 and 3 M GdnHCl, respectively. The data shown in Fig. 5 refer to samples assayed 2.5 h after refolding was initiated by dilution; however, further experiments showed that there was no variation in the % activity regained of samples taken at times from 5 min to 24 h after dilution. In a control experiment it was shown that the inclusion of dithiothreitol (1 mM) in the buffers used for denaturation and for subsequent dilution had no effect on the degree of reactivation when the enzyme had been denatured in 3 M GdnHCl. The inability to regain activity (Fig. 5) is thus not a consequence of oxidative damage to thiol groups in the enzyme. It should also be noted that the concentration of citrate synthase during attempted refolding (4  $\mu\text{g}/\text{ml}$  or 80 nM subunits) is well within the range (1 nM to 1  $\mu\text{M}$ ) of concentrations used for the successful reactivation of a number of oligomeric enzymes [20]. It is thus unlikely that the lack of reactivation in this case is due to a low concentration of enzyme during attempted refolding.

#### Reactivation of citrate synthase after exposure to high pH

As mentioned in the Introduction, it has been reported that citrate synthase can be dissociate into monomers at pH 9.6 and that partial reactivation occurs on readjustment of the pH to 8.0 [12]. In the present work we have confirmed that partial reactivation (20% activity regained) does indeed occur by this procedure after 15 min incubation in 50 mM Tris-HCl (pH 9.6). From the far-UV CD spectrum, it is clear that exposure

to pH values as high as 10.0 causes relatively little unfolding of the protein, with  $< 15\%$  change in  $[\theta]_{225}$ . The pH-induced dissociation into subunits [12] is thus not accompanied by gross structural changes in the enzyme.

#### Discussion

The results reported in this paper show that citrate synthase is essentially completely unfolded by high ( $\geq 4$  M) concentrations of GdnHCl and that little, if any, reactivation occurs on dilution of the denaturing agent. As illustrated in fig. 4c, the loss in enzyme activity occurs at lower concentrations of GdnHCl than the structural changes detected by thiol group reactivity, fluorescence or CD. This situation has also been reported in a number of other enzymes, and is generally interpreted in terms of the greater flexibility and hence higher sensitivity to denaturants of the active site region compared with the molecule as a whole [21]. The changes in the overall secondary and tertiary structures of citrate synthase run broadly in parallel (Fig. 4c), unlike the situation in aspartate aminotransferase isoenzymes [8,9], but similar to yeast phosphoglycerate kinase [22].

As indicated in fig. 5, there is partial reactivation of citrate synthase previously incubated in GdnHCl, at concentrations below those which cause complete unfolding. A comparison of the data shown in Fig. 5 with those in Figs. 2, 3 and 4 indicates that it is only that proportion of the enzyme molecules which retain native or native-like structure which can be reactivated on dilution of the denaturing agent. The results obtained on the enzyme when it is exposed to high pH are consistent with this conclusion; thus there is only a relatively small loss in secondary structure and partial reactivation can be observed on readjustment of the pH to 8.0.

Citrate synthase is thus a further example in addition to mitochondrial aspartate aminotransferase [9] and glutamate dehydrogenase [7] of a translocated protein which is unable to refold from its isolated, unfolded subunits. The mechanism of folding and assembly of such proteins *in vivo* raises a number of interesting questions, since several lines of evidence [23,24] have shown that translocated proteins cross the mitochondrial or other membranes in a non-native, or unfolded state. Although it is possible that folding could be more efficient at the higher concentrations of protein likely to occur *in vivo* than in the present studies, it is probable that folding and assembly of these imported proteins involves the participation of specific binding (or 'chaperone') proteins [10,25] which have been implicated in the assembly of ribulose biphosphate carboxylase-oxygenase within the chloroplast. In support of this hypothesis it has been recently shown that one such binding protein (the mitochondrial heat shock

protein hsp 60) is essential for the assembly of various oligomeric enzymes (such as  $F_1$ -ATPase) imported into yeast mitochondria [26]. The mechanism by which such proteins might play this role requires further investigation.

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# The unfolding and refolding of pig heart fumarase

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The unfolding of pig heart fumarase in solutions of guanidinium chloride (GdnHCl) has been examined. Loss of activity occurs at lower concentrations of GdnHCl than the structural changes detected by fluorescence or c.d. After denaturation, regain of activity can be observed provided that a reducing agent (dithiothreitol) is present and that the concentration of GdnHCl is lowered by dialysis rather than by dilution. The regain of secondary structure occurs with high efficiency even when little or no activity is recovered.

## INTRODUCTION

Fumarase (fumarate hydratase, EC 4.2.1.2) from pig heart is a tetrameric enzyme of  $M_r$  194 000 (Beeckmans & Kanarek, 1977). The enzyme is thought to occur in the matrix of the mitochondria, although there is some evidence that it, along with other tricarboxylic acid-cycle enzymes, may bind to the mitochondrial inner membrane (Srere, 1982). By using covalently immobilized enzyme, evidence has been found for interactions between fumarase and other enzymes of the cycle, notably malate dehydrogenase and citrate synthase (Beeckmans & Kanarek, 1981).

Like most mitochondrial enzymes, fumarase is synthesized as a precursor in the cytosol and subsequently imported into the organelle. As part of a series of experiments in which we have studied the folding and assembly of several mitochondrial enzymes (West & Price, 1988, 1990; West *et al.*, 1990), we have investigated the unfolding and refolding of fumarase. In earlier work (Teipel & Koshland, 1971; Teipel & Hill, 1971), it had been reported that some regain of fumarase activity after denaturation was observed on dilution of guanidinium chloride (GdnHCl) and that this could be enhanced by inclusion of substrate (L-malate). However, in view of the reported improvement in stability of the enzyme prepared by affinity chromatography (Beeckmans & Kanarek, 1977), it seemed worthwhile to investigate the unfolding and refolding of fumarase in more detail. In this paper, we report that loss of fumarase activity occurs at much lower concentrations of GdnHCl than the structural changes observed by fluorescence or c.d. Regain of activity after denaturation is possible provided that dithiothreitol is present, and that the concentration of GdnHCl is lowered by dialysis rather than by dilution. The structural changes on refolding have also been examined.

## EXPERIMENTAL

Pig heart fumarase, prepared by the method of Beeckmans & Kanarek (1977), was purchased as an  $(\text{NH}_4)_2\text{SO}_4$  suspension from Sigma. The preparation was at least 95% homogeneous as judged by Coomassie Blue staining after SDS/PAGE (Laemmli, 1970). The subunit  $M_r$  (48 000) was in agreement with previous determinations (Beeckmans & Kanarek, 1977).

Before use, the enzyme was dialysed extensively against 50 mM-sodium phosphate buffer, pH 7.3. When necessary, the concentration of the enzyme was increased by dialysis against a 20% (w/v) solution of poly(ethylene glycol) ( $M_r$  approx. 8000) in 50 mM-sodium phosphate buffer, pH 7.3. Poly(ethylene glycol) was purchased from Sigma.

The concentrations of solutions of enzyme were determined by the Coomassie Blue binding method (Sedmak & Grossberg, 1977), which was found to be more reliable and sensitive than spectrophotometric determination at 280 nm. The validity of the dye-binding method was confirmed by amino acid analysis by using the published amino acid composition data (Beeckmans & Kanarek, 1977). Kanarek & Hill (1964) had previously mentioned the problems caused by turbidity and light-scattering in the accurate spectrophotometric determination of fumarase concentrations.

Enzyme activity was assayed spectrophotometrically at 250 nm by using a volume of 1 ml of 50 mM-L-malate in 50 mM-sodium phosphate buffer, pH 7.3, at 25 °C. In terms of the units described by Kanarek & Hill (1964), the specific activity of the enzyme was found to be 31 500 units/mg, in close agreement with the value (33 000 units/mg) quoted by Beeckmans & Kanarek (1977). Our value for the specific activity corresponds to 390  $\mu\text{mol}$  of fumarate formed/min per mg of protein (Bock & Alberty, 1953).

c.d. spectra were recorded at 20 °C on a JASCO J-600 spectropolarimeter. Molar ellipticity values were obtained by using a value of 110 for the mean residue weight (Beeckmans & Kanarek, 1977). The secondary-structure content was determined by applying the CONTIN procedure at 1 nm intervals over the range 190–240 nm (Provencher & Glöckner, 1981).

Fluorescence spectra were recorded at 20 °C in a Perkin-Elmer MPF3A fluorimeter.

GdnHCl (Ultrapure grade) was purchased from Gibco BRL, Paisley, Scotland, U.K. The concentrations of solutions were checked by refractive-index measurements (Nozaki, 1972).

## RESULTS

### Secondary structure of fumarase

The far-u.v. c.d. spectrum of pig heart fumarase is shown in Fig. 1. As determined by the CONTIN procedure (Provencher & Glöckner, 1981) over the range 190–240 nm, the percentages of  $\alpha$ -helix and  $\beta$ -sheet structure are  $57 \pm 1.6$  and  $26 \pm 2.2$  respectively. Previous optical-rotatory-dispersion measurements had been interpreted in terms of an  $\alpha$ -helical content of about 50% (Kanarek & Hill, 1964). At present there are no high-resolution X-ray structural data on fumarase with which to compare these estimates.

### Unfolding of fumarase by GdnHCl

The unfolding of the enzyme was monitored in a variety of ways.

Abbreviation used: GdnHCl, guanidinium chloride.

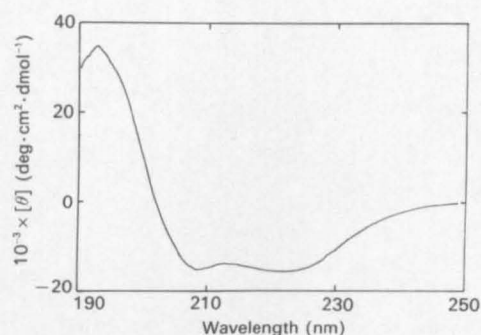


Fig. 1. Far-u.v. c.d. spectrum of fumarase

The spectrum was recorded at 20 °C in 50 mM-sodium phosphate buffer, pH 7.3. The enzyme concentration was 50  $\mu$ g/ml and the path-length 0.1 cm.

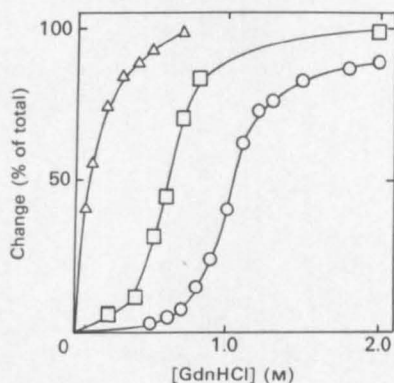


Fig. 2. Unfolding of fumarase by GdnHCl

Unfolding was monitored by changes in ( $\Delta$ ) enzyme activity, ( $\square$ ) fluorescence at 325 nm and ( $\circ$ ) ellipticity at 225 nm as described in the text. Solutions were incubated for 15 min at 20 °C in 50 mM-sodium phosphate buffer, pH 7.3, containing 1 mM-dithiothreitol in the presence of the indicated concentration of GdnHCl. Concentrations of enzyme were ( $\Delta$ ,  $\circ$ ) 30  $\mu$ g/ml and ( $\square$ ) 23  $\mu$ g/ml. In each case the changes are expressed relative to the total change observed between 0 and 4 M-GdnHCl, at which point the changes were essentially complete.

**Loss of activity.** Fumarase (30  $\mu$ g/ml) was incubated at 20 °C in the presence of GdnHCl in 50 mM-sodium phosphate buffer, pH 7.3, containing 1 mM-dithiothreitol; samples (20  $\mu$ l) were removed for assay, with the same concentration of GdnHCl present in the assay mixture as in the original incubation. The loss of activity after 15 min incubation as a function of [GdnHCl] is shown in Fig. 2. From these data it is clear that the integrity of the active site is very easily disturbed by low concentrations of the denaturing agent (i.e. 0.1 M-GdnHCl causes 55% loss of activity). If GdnHCl is not included in the assay mixture, the losses of activity are much less. Thus, for example incubation with 0.5 M-GdnHCl for 15 min causes 41% and 93% losses of activity when GdnHCl is absent from or present in the assay mixture respectively. This difference is presumably due to rapid refolding in the assay mixture of at least a portion of the denatured fumarase in the former case.

**Changes in fluorescence.** When excited at 290 nm, fumarase shows an emission maximum at 325 nm characteristic of tryptophan side chains buried in the interior of the protein, away from the aqueous solvent. On addition of GdnHCl there is a decrease in the fluorescence and a shift of the emission maximum towards the red. Thus in the presence of 4 M-GdnHCl, the emission

maximum was at 353 nm, a value characteristic of tryptophan side chains fully exposed to the solvent, and the fluorescence at 325 nm was 25% that of the enzyme in the absence of GdnHCl. The changes in fluorescence at 325 nm after 15 min incubation in GdnHCl solutions containing 1 mM-dithiothreitol are shown in Fig. 2.

**Changes in far-u.v. c.d.** When fumarase was incubated in the presence of increasing concentrations of GdnHCl containing 1 mM-dithiothreitol, there was a decline in the far-u.v. c.d. signal, reflecting the loss of secondary structure. The changes in  $[\theta]_{225}$  (which broadly measure  $\alpha$ -helical content) after 15 min incubation are shown in Fig. 2.

**Changes in quaternary structure.** The quaternary structure of fumarase was monitored by gel filtration on Sepharose 4B (under the conditions described in the legend to Fig. 5). In the absence of GdnHCl, the elution volume of fumarase was 18 ml; this was increased to 23 ml in the presence of 0.5 M-GdnHCl. The latter value corresponded to the elution volume of yeast phosphoglycerate kinase ( $M_r$  45000), indicating that at this concentration of GdnHCl fumarase is essentially dissociated into subunits. At higher concentrations of GdnHCl, where loss of secondary and tertiary structure is more evident, the behaviour of fumarase on gel filtration was more difficult to interpret. Thus, for instance, in the presence of 2 M-GdnHCl the elution volume was 19 ml. It is likely that, under such conditions, the behaviour reflects the extensive unfolding of the subunits, leading to a decrease in elution volume compared with that of a compact globular structure (Andrews, 1970; Mann & Fish, 1972).

**Comparison of the various measurements of unfolding.** From Fig. 2 it is clear that the changes in activity in the enzyme are observed at much lower concentrations of denaturing agent than are the changes in tertiary structure (as judged by fluorescence), which in turn occur at lower concentrations of GdnHCl than the changes in secondary structure (far-u.v. c.d.). These observations are consistent with the pattern noted previously for a number of enzymes, including creatine kinase and glyceraldehyde-3-phosphate dehydrogenase (Tsou, 1986).

**Time-dependence of unfolding.** The data shown in Fig. 2 refer to measurements made after 15 min incubation in the presence of various concentrations of GdnHCl. At concentrations of GdnHCl of 0.4 M and below, or 1 M and above, there was no detectable time-dependence of the values of the various parameters depicted in Fig. 2 (i.e. the values observed after 1 min and 60 min incubation did not differ significantly).

In this 'intermediate' region of [GdnHCl], however, time-dependent changes were observed in the various measurements of unfolding, although it should be noted that the ranges of concentration of GdnHCl at which such effects were observed were not identical for all three parameters. Changes in activity and fluorescence were observed, for instance, at 0.5 M- and 0.7 M-GdnHCl; however, the changes in c.d. were not seen at the lower of these concentrations. Selected data are shown in Fig. 3. In each case the changes are complete within 30 min, and no additional change is seen over a further 90 min.

There was no significant effect of omitting dithiothreitol from the incubation mixtures on the measurements of unfolding of the enzyme.

#### Refolding of fumarase after denaturation in GdnHCl

After incubation for 15 min in GdnHCl, the refolding of fumarase was attempted either by dilution into or by dialysis against 50 mM-sodium phosphate buffer, pH 7.3. In the dilution

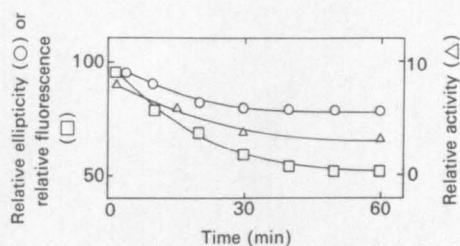


Fig. 3. Time-dependence of unfolding of fumarase by intermediate concentrations of GdnHCl

( $\Delta$ ), ( $\square$ ) and ( $\circ$ ) refer to changes in enzyme activity, fluorescence and ellipticity as described in Fig. 2. In each case the value corresponding to the enzyme in the absence of GdnHCl is 100. Note the different scale used for the values of enzyme activity.

procedure the residual GdnHCl was 0.02 M; in the dialysis procedure the residual concentration was shown by refractive-index measurements to be less than 0.01 M.

The results of applying the dilution procedure are shown in Fig. 4. It is clear that (a) there is a marked inability of the enzyme to regain activity when the initial concentration is of greater than about 1 M, and (b) omission of dithiothreitol leads to a lower regain of activity. The extent of regain of activity could not be increased by increasing the time of incubation or by increasing the concentration of enzyme in the refolding mixture. Essentially similar data to those shown in Fig. 4 were observed when samples were assayed after 24 h incubation in the refolding mixture, or when the final concentration of enzyme in this mixture was varied from 1 to 17  $\mu\text{g}/\text{ml}$ . In addition, there was no effect of including L-malate (5 mM) in the refolding mixture (Fig. 4).

When fumarase denatured in GdnHCl was subjected to dialysis to remove the denaturant, a substantial proportion of activity could be regained (Table 1). From Table 1 it is clear that the presence of dithiothreitol is crucial to this regain. A further set of experiments showed that the presence of the reducing agent was necessary in the refolding step (i.e. during dialysis) rather than in the denaturation step, and that L-malate (5 mM) could not

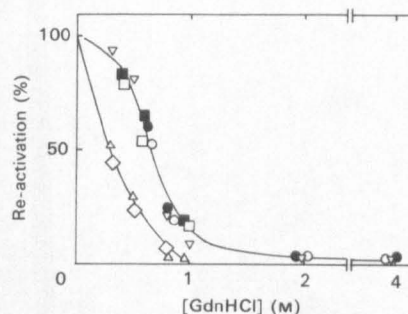


Fig. 4. Re-activation of fumarase after unfolding by GdnHCl

Enzyme was incubated for 15 min at 20 °C in 50 mM-sodium phosphate buffer, pH 7.3, containing 1 mM-dithiothreitol, then diluted into buffer at 20 °C containing dithiothreitol to give a residual concentration of GdnHCl of 0.02 M. Enzyme activity was measured 1 h after dilution. In each case re-activation is expressed relative to a control sample incubated in the absence of GdnHCl. ( $\nabla$ ), ( $\circ$ ) and ( $\square$ ) refer to enzyme concentrations (after dilution) of 2.5, 5 and 13  $\mu\text{g}/\text{ml}$  respectively, and ( $\bullet$ ) and ( $\blacksquare$ ) to 5 and 13  $\mu\text{g}/\text{ml}$  respectively with 5 mM-L-malate included in the dilution buffer. ( $\Delta$ ) and ( $\diamond$ ) refer to enzyme concentrations of 2.5 and 13  $\mu\text{g}/\text{ml}$  respectively with no dithiothreitol included in either the unfolding or re-activation steps.

substitute for dithiothreitol in bringing about re-activation. Essentially similar data were obtained when the concentration of enzyme was 20 or 10  $\mu\text{g}/\text{ml}$ , i.e. within the range of concentrations where the dilution procedure did not lead to regain of activity at an initial [GdnHCl] > 1 M.

From the data in Table 1, the regain of secondary structure (far-u.v. c.d.) is seen to be almost 100% efficient under all circumstances. However, the regain of tertiary structure (as judged by both the emission maximum and intensity) is much less complete in the absence of dithiothreitol than in its presence, and this behaviour is found to parallel the regain of activity. This implies that the integrity of thiol groups is required for correct folding and that this integrity is very easily damaged in the unfolded state.

Table 1. Refolding of fumarase after denaturation in GdnHCl

Denaturation was performed by incubating the enzyme for 15 min at 20 °C with the stated concentrations of GdnHCl and dithiothreitol. Dialysis was performed at 4 °C for 18 h against 500 vol. of 50 mM-sodium phosphate buffer, pH 7.3. The final concentration of enzyme was 30  $\mu\text{g}/\text{ml}$ . Values shown are the means of duplicate measurements with less than 10% variation in the values of activity, fluorescence ( $F_{325}$ ) and molar ellipticity. The precision of the emission maximum is estimated at  $\pm 1$  nm.

Sample	Activity (% of native)	$F_{325}$ (% of native)	Emission maximum (nm)	$[\theta]_{225}$ (% of native)
Native enzyme	100	100	325	100
Enzyme + 1.5 M-GdnHCl + 1 mM-DTT*				
Before dialysis	0	28	345	22
After dialysis	62	94	325	98
Enzyme + 1.5 M-GdnHCl				
Before dialysis	0	28	345	22
After dialysis	3	72	333	96
Enzyme + 4 M-GdnHCl + 1 mM-DTT*				
Before dialysis	0	25	353	8
After dialysis	48	79	325	95
Enzyme + 4 M-GdnHCl				
Before dialysis	0	25	353	8
After dialysis	1	67	335	96

\* 1 mM-dithiothreitol (DTT) was included in both the unfolding incubation mixture and the buffer against which the sample was dialysed.

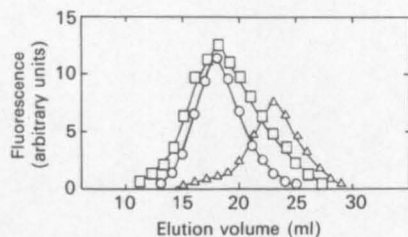


Fig. 5. Gel filtration of native and refolded fumarase

Samples (1 ml) of enzyme (approx. 30  $\mu\text{g}/\text{ml}$ ) were applied to a column (1.8  $\text{cm}^2 \times 10 \text{ cm}$ ) of Sepharose 4B eluted with 50 mM-sodium phosphate buffer, pH 7.3; 1 ml fractions were collected. Protein was monitored by fluorescence with excitation and emission wavelengths of 290 and 325 nm respectively. (○) refers to native enzyme; (△) and (□) refer to enzyme denatured by 4 M-GdnHCl and refolded by dialysis in the absence and presence of 1 mM-dithiothreitol respectively. In a separate experiment, the elution volume of yeast phosphoglycerate kinase was determined to be 23 ml.

The ability of the enzyme to regain its quaternary structure after refolding was examined by gel filtration on Sepharose 4B. As shown in Fig. 5, enzyme which had been denatured and refolded in the presence of dithiothreitol had regained the native tetrameric structure, as judged by its elution volume (18 ml). By contrast, enzyme which had been denatured and refolded in the absence of dithiothreitol and which had regained little, if any, activity (Table 1) was eluted at 23 ml, corresponding to the position of elution of yeast phosphoglycerate kinase ( $M_r$  45000). This indicates that under these conditions the enzyme remains in a monomeric state after dialysis.

## DISCUSSION

In this paper we have demonstrated that the changes in catalytic activity, secondary structure and tertiary structure of fumarase occur over different concentration ranges of GdnHCl (Fig. 2). This is similar to the type of behaviour reported (Tsou, 1986) for other enzymes and is taken to reflect the sensitive nature of the structure of the active site. The results of gel filtration indicate that in the presence of 0.5 M-GdnHCl, the enzyme is dissociated into subunits, although no great loss of native secondary or tertiary structure has occurred. These results indicate that the catalytic activity of fumarase depends on the tetrameric structure of the enzyme, a conclusion consistent with that reached by Teipel & Hill (1971) using sedimentation-velocity measurements under somewhat different conditions. On refolding, there is efficient regain of secondary structure and the regain of tertiary structure is more marked than that of catalytic activity (Table 1); this is in accord with current models of protein folding (Jaenicke, 1987; Creighton, 1990), in which formation of secondary structure precedes that of 'compact intermediate' and finally catalytically active states.

We have been able to observe some of the features previously reported for the unfolding and refolding of fumarase (Teipel & Hill, 1971; Teipel & Koshland, 1971). Thus we have observed a time-dependence of inactivation at intermediate concentrations of GdnHCl (Fig. 3). Teipel & Hill (1971) noted that activity was slowly lost at concentrations of GdnHCl less than 0.5 M. However, they observed much less inactivation than in the present paper, presumably because they did not include GdnHCl in the assay mixture, and this allowed at least partial refolding to occur before the rate of product formation was recorded. Like Teipel & Hill (1971), we have observed that dialysis of the denatured enzyme against a buffer containing a reducing agent can lead to substantial regain of activity (Table 1). However, unlike Teipel &

Koshland (1971), we have been unable to observe re-activation by dilution of the denatured enzyme with initial GdnHCl concentrations above 2 M or to observe any enhancing effect of L-malate on this re-activation. It is possible that the differences between our data and the earlier results may arise from small differences between the enzyme preparations, which, as noted by Beeckmans & Kanarek (1977), are reflected in slight differences in specific activity and composition and pronounced differences in stability on incubation at pH 7.3.

Slow structural transitions at intermediate concentrations of denaturant (Fig. 3) have been observed in other systems (see, e.g., Zettlmeissl *et al.*, 1981; Tsou, 1986). The nature of these transitions remains to be explored, although clearly relatively small structural rearrangements are involved. In some cases *cis*  $\rightleftharpoons$  *trans* isomerization of proline peptide bonds may also occur (Zettlmeissl *et al.*, 1981; Brandts & Lim, 1986). Presumably in the presence of these intermediate concentrations of denaturing agents a number of conformational states are kinetically accessible to the enzyme.

In common with other mitochondrial enzymes which we have studied, e.g. glutamate dehydrogenase (West & Price, 1988), aspartate aminotransferase (West & Price, 1990) and citrate synthase (West *et al.*, 1990), fumarase did not regain activity on dilution of the denatured enzyme, provided that the initial concentration of GdnHCl was higher than about 1.5–2 M. For fumarase, the ability to regain activity ran roughly in parallel with the changes in tertiary structure detected by fluorescence (compare Figs. 2 and 4). However, using the dialysis procedure to lower the GdnHCl concentration led to substantial re-activation. The difference between the two methods is not due to the different temperatures used, since a control experiment showed that dilution into buffer at 4 °C led to no increase in re-activation compared with 20 °C. There has been little systematic study of the different procedures used to bring about refolding of denatured proteins, but it is probable that the success of the dialysis procedure in the present case arises from the gradual lowering of the GdnHCl concentration so that 'correct' structural adjustments can occur. By contrast, when the concentration of GdnHCl is lowered suddenly (e.g. by dilution), the enzyme may be prevented from acquiring its proper tertiary structure by kinetic-energy barriers. A number of examples of irreversible unfolding of proteins at critical concentrations of denaturants have been reported (see, e.g., London *et al.*, 1974; Ghélis & Yon, 1982; Mitraki *et al.*, 1987).

The relevance of the findings reported in this paper to the folding and assembly of fumarase *in vivo* remains to be established. The dependence of the re-activation on the refolding conditions implies that special mechanisms may exist to ensure correct folding *in vivo*. In the light of current data on the import and assembly of mitochondrial and chloroplast proteins, it is possible that 'chaperone' proteins may play a role in this folding process (Cheng *et al.*, 1989; Ellis & Hemmingsen, 1989; Ostermann *et al.*, 1989).

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# The aromatic amino acid content of the bacterial chaperone protein groEL (cpn60)

## Evidence for the presence of a single tryptophan

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Studies of the absorption and fluorescence properties of the chaperone protein groEL (cpn60) from *Escherichia coli* show that tryptophan is present. In contrast to the proposed amino acid sequence of the protein (Hemmingsen, S.M. et al. (1988) *Nature* 333, 330–334). By determining a suitable value for the specific absorption coefficient of the protein at 280 nm, it has been shown that the content of the aromatic amino acids corresponds to a single tryptophan and (most probably) seven tyrosines per subunit (*M*, 57 200).

Chaperone protein; Fluorescence; Spectrophotometry

### 1. INTRODUCTION

The proteins encoded by the *groE* gene of *E. coli* were discovered as critical components for the assembly of large bacteriophages such as lambda and T4 [1,2]. These proteins, groEL, a tetradecamer of subunit *M*, 57 200 (also known as cpn60), and groES, a heptamer of subunit *M*, 10 300 (also known as cpn10) have been purified [3,4]. They are the subject of much current research designed (i) to establish the way in which they assist in the correct folding and assembly of other proteins, and (ii) to explore the relationships between the various groups of chaperone proteins (for recent reviews, see for example [5–7]). The amino acid sequences (derived from the DNA sequence) of the groEL and groES proteins have been published [8]. This work indicates that the groEL protein contains 7 tyrosines and no tryptophan per subunit. However, earlier amino acid composition data [3] had indicated that tryptophan was present in small amounts (0.19 mol% or approximately 1 per subunit) in the groEL protein. In addition values quoted for the specific absorption coefficient of the groEL protein at 280 nm (using either nitrogen analysis [9] or amino acid analysis [10]) are significantly different from each other, and are both incompatible with the proposed amino acid sequence data (see section 4).

**Abbreviations:** GdnHCl, guanidinium chloride; c.d., circular dichroism.

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In view of the well known difficulties in amino acid analysis in the quantitative estimation of tryptophan in proteins, because of the lability of the amino acid on hydrolysis, we have used the more sensitive spectrophotometric (absorption and fluorescence) techniques to show conclusively that tryptophan is present in groEL preparations. By establishing a suitable value for the specific absorption coefficient of the protein at 280 nm, we are able to show that the content corresponds to a single tryptophan per subunit. This information is of interest, not only because of the need to establish protein concentrations in order to calculate, for example, stoichiometries of association with other proteins, but also because the intrinsic fluorescence of groEL should be taken into account when fluorescence is used to monitor the folding of a target protein.

### 2. EXPERIMENTAL

The groEL protein was purified from *E. coli* strain MC1061 carrying the plasmid pND5 described by Jenkins et al. [11] using methods described by Hendrix [3,4]. The product eluted as a single symmetrical peak (of high *M*) on gel filtration on Superose 6, on anion-exchange chromatography at pH 7.5 on MonoQ and was at least 98% homogeneous as judged by Coomassie blue staining following SDS-PAGE on 12% acrylamide gels [12]; the subunit *M*, was 56 500 ± 2000, consistent with the published value [8].

GdnHCl (ultrapure grade) was purchased from Gibco BRL, Paisley, Scotland, UK. The concentrations of solutions of GdnHCl were checked by refractive index measurements [13].

Fluorescence measurements were made at 20°C on a Perkin Elmer LS50 spectrofluorimeter. C.d. spectra were recorded at 20°C on a JASCO J600 spectropolarimeter; determination of secondary structure was undertaken using the CONTIN procedure [14]. For these measurements, the groEL protein was in the buffer system used by

Lissin et al. [9] in their studies of the self assembly of groEL, namely 50 mM potassium phosphate, 20 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM dithiothreitol, pH 7.5.

Determinations of protein concentrations were undertaken using a variety of methods: (i) far u.v. measurements [15], (ii) Coomassie blue binding [16], (iii) bicinchoninic acid [17], (iv) amino acid analysis. Methods (ii) and (iii) were performed with reference to bovine serum albumin as standard. Method (iii) was performed using the buffer noted above but without dithiothreitol. Method (i) could not be performed using this buffer, because of the high absorption due to acetate in the far u.v.; measurements were therefore made in 50 mM sodium phosphate, pH 7.5. There was no detectable difference in the absorbance at 280 nm of a solution of groEL when these different buffers were used. Amino acid analysis was performed at the WELMET Protein Characterisation Facility at the University of Edinburgh. All spectrophotometric measurements were performed at least 5 times, and the amino acid analysis was performed in duplicate. The error in the various determinations is estimated to be less than  $\pm 5\%$ .

### 3. RESULTS

#### 3.1. Evidence for the presence of tryptophan in the groEL protein

##### 3.1.1. Measurements of absorbance

The ratio of absorbances of a protein at 280 and 288 nm depends critically on the content of tyrosine and tryptophan in the protein [18]. Using the published values for these amino acids at these wavelengths [18], it is possible to calculate the ratio for a protein in the presence of 6M GdnHCl. If tryptophan were absent and only tyrosine present, as in the published amino acid sequence [8], the ratio should be 3.325. If tryptophan is present, the ratio is lowered (for tryptophan itself the ratio is 1.182). From seven separate determinations for the groEL protein in the presence of 6 M GdnHCl, we find a ratio of  $1.96 \pm 0.05$ . (It should be noted that the value is slightly lower ( $1.83 \pm 0.05$ ) in the absence of GdnHCl, reflecting environmental effects on the chromophores in the intact protein.) The ratios calculated for various combinations of ratios of tryptophan (W) and tyrosine (Y) are, for example, 1.951 (1W + 7Y); 2.018 (1W + 8Y); 1.650 (2W + 7Y) (these data refer to a protein in the presence of 6 M GdnHCl). Thus, the observed results clearly indicate the presence of tryptophan in the groEL protein.

##### 3.1.2. Fluorescence measurements

The emission wavelengths for tryptophan and tyrosine derivatives are about 355 and 305 nm, respectively [19]. In proteins, there can be shifts from these values because of environmental effects; these effects can be particularly marked in the case of tryptophan side chains where emission maxima lower than 325 nm have been reported [19,20]. The fluorescence spectrum of the groEL protein in buffer (excited at 280 nm) is shown in Fig. 1 (curve A). There is a peak at about 310 nm due predominantly to tyrosine side chains, but significant fluorescence is still observed at wavelengths up to about 400 nm. The spectrum is difficult to interpret unequivocally,

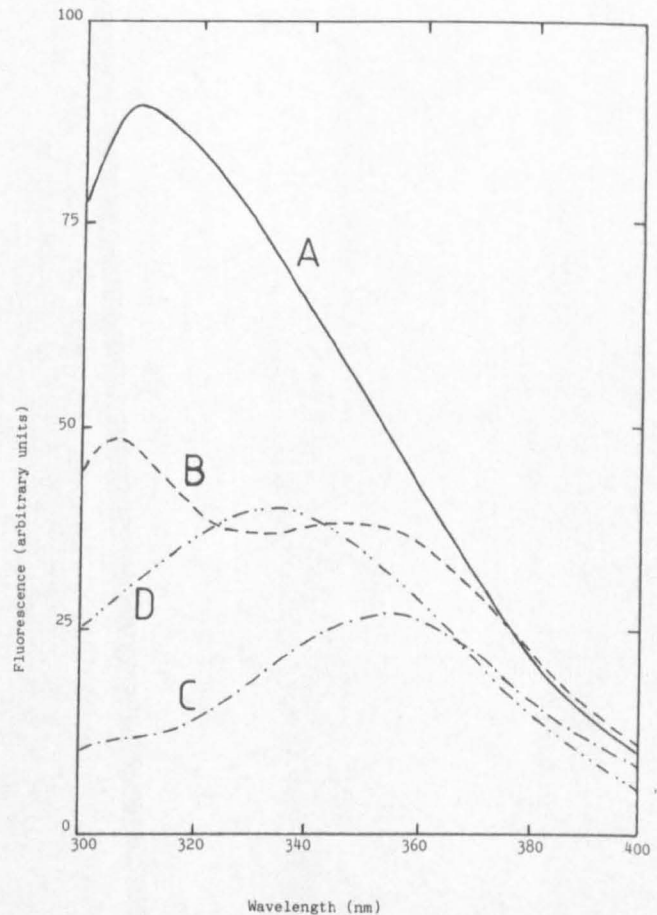


Fig. 1. Fluorescence emission spectra of groEL protein. Spectra were recorded at 20°C in 50 mM potassium phosphate, 20 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM dithiothreitol, pH 7.5. The protein concentration was 61  $\mu\text{g/ml}$ . Curve A, excitation at 280 nm; curve B, as A, but in the presence of 6 M GdnHCl; curve C, as B, but with excitation at 290 nm; curve D, as curve A, but with excitation at 290 nm. Spectra were corrected for the effects of Raman scattering.

because of the environmental effects noted above. The situation is made much clearer when (i) the groEL protein is denatured in 6 M GdnHCl, so that the environmental effects are essentially removed, and (ii) excitation is performed at 290 nm, where tryptophan absorbs much more strongly than tyrosine. Under these circumstances, the fluorescence spectra show distinct maxima at 355 nm (Fig. 1, curves B and C) characteristic of tryptophan emission. Curve D in Fig. 1 shows the spectrum obtained by exciting at 290 nm in the absence of GdnHCl; the emission maximum is 334 nm, corresponding to a moderate degree of exposure of a tryptophan side chain [19,20].

#### 3.2. The specific absorption coefficient of the groEL protein at 280 nm

Using the various methods outlined in the Experimental section, values for the specific absorption coefficient of the groEL protein at 280 nm were determined.

(i) Far u.v. measurements [15]. In 50 mM sodium phosphate buffer, pH 7.5, we find that the absorbance

of the groEL protein at 205 nm is 88.1 times that of the absorbance at 280 nm. In order to evaluate the molar absorption coefficient of the groEL protein at 205 nm, it is necessary to consider the chromophores concerned [15]. The major contribution is made by the peptide bond; from the molar coefficient (2400 [15]), this is calculated to represent 22.95 for a 1 mg/ml solution of the groEL protein assuming the published values [8] for the number of amino acids and the subunit  $M_r$ . Smaller contributions are made by the side chains of tyrosine, tryptophan, phenylalanine, histidine, arginine and methionine. Using the absorption coefficients for these amino acids [15] and their percent occurrence either from the sequence data [8] or the composition data [3], values of 26.43 or 26.21, respectively, can be calculated for the absorbance of a 1 mg/ml solution of the groEL protein at 205 nm. Using an average value of 26.32, the value of the absorbance at 280 nm for a 1 mg/ml solution is 0.299.

(ii), (iii) The methods involving Coomassie blue [16] or bicinchoninic acid [17] are comparative, in the sense that a standard protein (bovine serum albumin) is used. However, as discussed elsewhere [17,21], they are reasonably reliable measures of protein concentration, since they reflect the occurrence of positively charged side chains and peptide bonds, respectively, and in both respects the properties of the groEL protein are reasonably typical. The values obtained by the two methods for the absorbance at 280 nm of a 1 mg/ml solution of the groEL protein are 0.305 and 0.282, respectively.

(iv) Amino acid analysis. In order to relate the results of amino acid analysis to the content of protein, it is necessary to have accurate composition data. As mentioned in section 4, there is very close overall agreement between the results of direct amino acid analysis [3] and DNA (and hence amino acid) sequencing [8]. In particular, the data for the contents of lysine and arginine agree within 5%. These amino acids were chosen for analysis in view of their stability upon hydrolysis and their relative abundance in the groEL protein (7.3 and 4.0 mol%, respectively). On the basis of the amino acid analysis, the absorbance at 280 nm of a 1 mg/ml solution of groEL protein in buffer is 0.280 (based on the lysine content) or 0.275 (based on the arginine content). The tyrosine content was found to be 7.0 per subunit, a value consistent with the amino acid sequence (7) [8] and amino acid analysis (7.55) [3] information.

Taking all the values above into account, an average value of 0.288 for the absorbance at 280 nm of a 1 mg/ml solution of groEL is obtained. This value corresponds reasonably closely with that reported by Lissin et al. [9], based on nitrogen analysis. These authors reported a value of 0.25 at 276.5 nm; this would correspond to a value of 0.242 at 280 nm. However, it is likely that Lissin et al. [9] based their calculations on nitrogen content of 16%, the typical value for proteins. In fact, because of the preponderance of lighter amino acids in

the groEL protein, the actual nitrogen content is 16.9%, which would have the effect of raising the absorbance value to 0.255. If this value is included along with our data, an average value of 0.283 is derived. In conclusion, we consider that a value of 0.285 for the specific absorption coefficient ( $\text{litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ ) at 280 nm is appropriate for the groEL protein.

### 3.3. Content of tryptophan and tyrosine in the groEL protein

Assuming the above value for the absorbance of the groEL protein at 280 nm, the content of the aromatic amino acids can be calculated. The absorbance at 280 nm in the presence of 6 M GdnHCl is found to be  $0.95 \pm 0.01$  times that in the absence of GdnHCl, i.e. 0.271 for a 1 mg/ml solution; at 288 nm, using the ratio determined earlier, the value is 0.138. Taking into account the reference values for tryptophan and tyrosine at these wavelengths [18], the numbers of these amino acids per subunit ( $M_r$  57 200) of the groEL protein are 1.04 and 7.47, respectively. Additional estimates of the numbers of these amino acids can be derived from fluorescence spectra in the presence of 6 M GdnHCl (see Fig. 1), using the *N*-acetyl amides of tryptophan and tyrosine as reference compounds. From five separate determinations the contents of these amino acids, based on the fluorescence at 355 and 305 nm, are 0.72 and 6.84 per subunit of the groEL protein, respectively. It is probably that the somewhat lower values than those determined by absorbance measurements reflect a degree of quenching by neighbouring portions of the polypeptide chain in the unfolded protein.

## 4. DISCUSSION

The data in this paper show that there is a single tryptophan and (most probably) seven tyrosines per subunit of the groEL protein. Whereas the latter value is in accord with the published amino acid sequence [8], the presence of tryptophan indicates that there is an error in the sequence data, arising presumably from a DNA sequencing error. The presence of tryptophan in the protein had been indicated by Hendrix [3], but the discrepancy with the sequence data had not been commented on. We believe that any DNA sequencing error(s) is (are) likely to be minor, since a comparison of the amino acid composition data determined directly [3] and by the DNA (and hence amino acid sequence) [8] using the method of Cornish-Bowden [22] shows that the compositions are very similar indeed, with a difference index ( $S\Delta n$ ) of only  $0.11N$ , where  $N$  is the number of amino acids in the polypeptide chain. In fact, the only serious deviations (outside 10%) are for histidine, cysteine and tryptophan, all of which are present in very small amounts.

It should be noted that amino acid sequences (derived from DNA sequences) have been determined for a num-

ber of groEL homologues in other organisms. A number, including *Mycobacterium leprae*, *M. tuberculosis*, *M. bovis* and human mitochondrial cpn60 show a conserved tryptophan at position 44 (groEL numbering), while the plastic derived products show a conserved tryptophan at position 475. Both of these regions of the *E. coli groE* gene have been sequenced more than once [8,23,24] and it seems unlikely that any error would be duplicated. An alternative explanation to a sequencing error is that a mutation has occurred leading to an amino acid change in the groEL protein. It would require careful sequence analysis of both the original gene and derived plasmids to exclude this possibility, but it should be noted that evidence from specific absorption values (see below) suggests that tryptophan is present in the groEL protein products of a number of different constructed plasmids [9,10].

The value for the specific absorption coefficient at 280 nm (0.285) for the groEL protein has been derived by a number of independent techniques, which, taken together, give a coherent picture. As further (albeit indirect) evidence for this value, it should be noted that when this value of the coefficient is used to calculate the mean residue ellipticities from the far u.v. c.d. spectrum, of the groEL protein, an  $\alpha$ -helical content of 56% is derived: this is close to the value obtained (57%) by applying the PREDICT set of programs [25] to the amino acid sequence of the protein, and to the value (57%) previously obtained by c.d. measurements [9].

It is of interest that the discrepancies between the observed absorption properties of the protein [9] and those predicted on the basis of the amino acid sequence [8] had not been commented on previously. If groEL contained seven tyrosines and no tryptophan per subunit, the absorbance of a 1 mg/ml solution at 280 nm can be calculated [18] as 0.157 (in 6 M GdnHCl), i.e. 0.165 in buffer. When a single tryptophan is included, the values become 0.256 and 0.270, respectively (very close to the values determined in this paper). The value reported by Viitanen et al. [10] for the absorption coefficient of the groEL protein at 280 nm ( $23800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ) would correspond to 0.416 for a 1 mg/ml solution; however, these authors gave relatively few details about the determination of this value, and made no comment on the discrepancy from the value predicted on the basis of the published amino acid sequence [8].

A final possible explanation for our results is that tryptophan is not present in the groEL protein, but is present in a contaminating protein in the preparation. We consider this unlikely in view of our analytical data on the preparation, since such a contaminant would either possess the same  $M_r$  as groEL under denaturing (SDS-PAGE) and non-denaturing (gel filtration) conditions or would be rich in tryptophan. (Assuming a 98% homogeneity value and a content of 0.19 mol% tryptop-

han overall, the contaminant would possess a tryptophan content of  $50 \times 0.19 \text{ mol}\%$ , i.e. 9.5 mol%.) Such a contaminant would be most unusual.

The data in this paper will help in the further characterisation of the groEL protein and its role in assisting protein folding and assembly, not only in calculating the concentration of the protein, but also in indicating the need to consider its intrinsic fluorescence when studying the folding of other proteins in reconstituted systems [25]. The location of the tryptophan residue in the protein will be undertaken by a combination of specific cleavage and sequencing methods, both at the DNA and protein level.

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### The refolding of mitochondrial proteins after denaturation

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Earlier work (reviewed in [1,2]) has shown that most cytoplasmic enzymes can be refolded after denaturation in guanidinium chloride (GdnHCl) with relatively high efficiency. Refolding is usually initiated by dilution of the solution so that the GdnHCl concentration is lowered from a value sufficient to cause unfolding (usually  $>4M$ ) to a value which has no detectable effect on the enzyme (usually  $\leq 0.1M$ ).

When this dilution procedure has been applied to a number of mitochondrial proteins, the yield of active enzyme has been very low ( $<5\%$ ). These enzymes include glutamate dehydrogenase [3], aspartate aminotransferase [4] and citrate synthase [5]. The case of aspartate aminotransferase is especially noteworthy since the cytoplasmic isoenzyme (which is similar in sequence and three-dimensional structure to the mitochondrial isoenzyme) can be refolded successfully by the dilution procedure provided that dithiothreitol and pyridoxal-5'-phosphate are present [6]. In recent work we have examined the behaviour of fumarase. Like the other mitochondrial enzymes, fumarase cannot be refolded by the dilution procedure after exposure to GdnHCl at concentrations above  $2M$  (a concentration which causes loss of secondary and tertiary structure of the enzyme as judged by circular dichroism and fluorescence respectively). However, if the solution of enzyme in  $4M$  GdnHCl is dialysed against buffer, substantial ( $50\%$ ) activity can be regained. This regain of activity is dependent on the presence of dithiothreitol; in the absence of the reducing agent  $<1\%$  activity is regained. The regain of activity is correlated with the regain of correct tertiary and quaternary structure, as judged by fluorescence and gel filtration respectively. By contrast the regain of secondary structure, as judged by far u.v. circular dichroism, occurs even when higher levels of structure and activity are not regained.

In preliminary experiments we have also been able to show that denatured citrate synthase can be reactivated by the dialysis procedure. The specific activity of the enzyme is about  $40\%$  that of the native enzyme, although some losses of soluble enzyme occur, probably due to aggregation. We are extending these observations to other mitochondrial enzymes.

It is probable that the success of the dialysis procedure is due to the gradual lowering of the concentration of GdnHCl, allowing a number of kinetically accessible conformations of the enzyme to interconvert during the search for the correct folding pathway. However, in the dilution procedure the rapid lowering of the GdnHCl concentration leads to the burying of hydrophobic side chains during the early phases of the folding process. The polypeptide chain could then be trapped in one or more conformations from which other conformations are not kinetically accessible.

In the cell, most mitochondrial proteins are imported after synthesis in the cytosol. The N-terminal targeting sequence is removed by one or more proteases in the mitochondrial matrix. Various lines of evidence suggest that the protein is in an unfolded or non-native state during translocation [7,8]. It is believed that chaperone proteins

(probably of the hsp 60 type) are involved in the correct folding and assembly of imported mitochondrial proteins [9,10] although the mechanism by which this occurs is not yet established. Our results indicate that the refolding conditions can affect the efficiency of regain of activity of denatured mitochondrial proteins. It is tempting to speculate that chaperone proteins may help to direct the correct folding of mitochondrial proteins by permitting the equilibration of various conformational states which act as intermediates in the folding pathway.

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## REACTIVATION OF DENATURED CITRATE SYNTHASE

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**Abstract**—1. The imported mitochondrial enzyme citrate synthase can be partially ( $\leq 45\%$ ) reactivated after denaturation in guanidinium chloride, if the concentration of the denaturing agent is lowered by dialysis, rather than by dilution, when essentially no reactivation is observed.

2. The presence of a reducing agent (dithiothreitol) is necessary for regain of activity.

3. Optimum regain of activity occurs at enzyme concentrations of about 10–20  $\mu\text{g/ml}$ ; at higher concentrations there is significant formation of aggregates.

### INTRODUCTION

In common with most mitochondrial proteins, citrate synthase [citrate oxaloacetate-lyase ([pro-3S]- $\text{CH}_2\text{COO}^- \rightarrow \text{acetyl-CoA}$ ), EC 4.1.3.7] is coded for by nuclear DNA and synthesised in the cytosol as a precursor which is subsequently translocated into the mitochondrion. As part of our investigations of the assembly of translocated proteins (West and Price, 1988, 1990; West *et al.*, 1990; Kelly and Price, 1991), we had previously shown that citrate synthase from pig heart which had been denatured by GdnHCl could not be reactivated by dilution of the denaturing agent (West *et al.*, 1990). This result has been subsequently confirmed in other studies (Buchner *et al.*, 1991), where it was shown that a limited degree ( $\leq 40\%$ ) of reactivation was possible, provided that the bacterial chaperone proteins GroEL and GroES together with ATP were added during the dilution step. The behaviour of the mitochondrial proteins is in contrast to that of the majority of cytosolic proteins, where significant reactivation after denaturation can be achieved by dilution (Jaenicke, 1987).

In this paper, we report that it is possible to achieve substantial reactivation of citrate synthase after denaturation in 6 M GdnHCl without the intervention of chaperone proteins, provided that the denaturing agent is removed by dialysis rather than by dilution. It is also necessary to include a reducing agent (dithiothreitol), presumably to protect cysteine side chains during the procedure.

### EXPERIMENTAL

Citrate synthase (pig heart) was purchased from Sigma. The preparation was at least 95% homogeneous as judged by Coomassie blue staining following SDS–polyacrylamide gel electrophoresis on 12% acrylamide gels (Laemmli, 1970). The subunit  $M_r$  was determined to be  $47,000 \pm 2500$ , compared with the  $M_r$  (48,969) calculated from the amino acid sequence (Bloxham *et al.*, 1982). Concentrations of enzyme were determined spectrophotometrically at 280 nm using

$A_{1\text{cm}}^{0.1\%} = 1.78$  (Bloxham *et al.*, 1980). At low concentrations of enzyme, the Coomassie blue binding method (Sedmak and Grossberg, 1977) was used to determine the concentration, because of its greater sensitivity; the method was calibrated using citrate synthase as a standard.

Citrate synthase was assayed spectrophotometrically as described previously (West *et al.*, 1990). Acetyl-CoA was prepared by acetylation of CoA as described (Stadtman, 1957). Control experiments showed that the residual concentrations ( $\leq 10 \mu\text{M}$ ) of dithiothreitol in the assay mixture did not interfere with the assay.

Citrate synthase was denatured by incubation in 6 M GdnHCl in 20 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol for 15 min at 20°C. Previous experiments (West *et al.*, 1990) had shown that this causes essentially complete loss of secondary and tertiary structure of the enzyme as judged by c.d. and fluorescence respectively. Refolding was performed by dialysis of the denatured enzyme (at 4 or 20°C) against two changes of 200 vol of 20 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol. Each dialysis was carried out for 3 hr. Measurements of refractive index (Nozaki, 1972) were used to check the concentrations of GdnHCl solutions. These confirmed that the residual concentrations of the denaturing agent after dialysis was  $< 0.01 \text{ M}$ . GdnHCl (Ultrapure grade) was purchased from Gibco BRL, Paisley, Scotland.

The recovery of protein and activity after the denaturation/refolding procedure was expressed in each case relative to a control sample from which GdnHCl had been omitted. In calculating recoveries, it was necessary to take into account the increase in volume of samples containing GdnHCl which occurs on dialysis; this increase was measured accurately in each case and was generally of the order of 25%.

c.d. Measurements were performed on a JASCO J-600 spectropolarimeter as described previously (West *et al.*, 1990).

### RESULTS

#### *Regain of activity after dialysis of denatured enzyme*

The extent of regain of activity of citrate synthase after dialysis is shown in Fig. 1. From these data it is possible to draw a number of conclusions.

(i) Reactivation is crucially dependent on the presence of reducing agent (dithiothreitol). It is therefore essential to maintain the integrity of the cysteine side chains (4 per subunit) during the unfolding and refolding of the enzyme.

**Abbreviations:** GdnHCl, guanidinium chloride; c.d. circular dichroism.

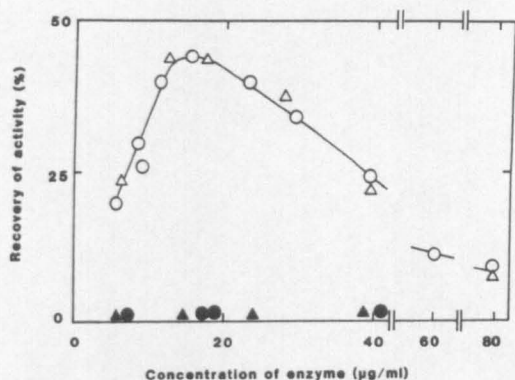


Fig. 1. Recovery of activity of citrate synthase after denaturation in 6 M GdnHCl. The concentration of enzyme shown on the abscissa is that before dialysis is performed. As explained in the text, the recovery of activity is calculated relative to a control sample from which GdnHCl is omitted, taking into account the increase in volume which occurs on dialysis. Open symbols ( $\circ$ ,  $\triangle$ ) or solid symbols ( $\bullet$ ,  $\blacktriangle$ ) refer to experiments in which 1 mM dithiothreitol is present or absent respectively. Circles and triangles refer to experiments performed at 4 and 20°C respectively.

(ii) The degree of reactivation depends on the concentration of enzyme which is dialysed. Optimum recoveries of about 45% activity are obtained at an initial concentration (i.e. before dialysis) in the range from about 10 to about 20  $\mu\text{g/ml}$ . (As explained in the Experimental section, dilution occurs on dialysis, but this is taken into account in calculating the recovery of activity). At concentrations above and below this range a decline in recovery of activity is observed.

A decrease in reactivation at low protein concentrations has been noted in other systems, and has been attributed to long-term instability of intermediates in refolding and/or nonspecific adsorption of protein to surfaces (Rudolph and Jaenicke, 1976; Gerschitz *et al.*, 1978; Hermann *et al.*, 1983). In view

of the data concerning recovery of soluble protein given below, the second possibility appears unlikely. The decline in reactivation at high concentrations of protein is most likely due to the competing effects of aggregation which, being of high kinetic order are favoured at high concentration (Jaenicke, 1982, 1987).

In our experiments there was evidence of significant turbidity and precipitation of protein at high concentrations. Buchner *et al.* (1991) have also drawn attention to the marked tendency of citrate synthase to form aggregates when the concentration of denaturing agent is lowered by dilution.

(iii) There is little or no difference between the recoveries of activity obtained by performing the dialysis at 4°C compared with 20°C. This lack of effect of temperature contrasts with the situation observed in the refolding of other denatured enzymes, e.g. rhodanese (Gatenby *et al.*, 1990) and dimeric ribulose 1,5-bisphosphate carboxylase (Viitanen *et al.*, 1990) where the efficiency of refolding is severely decreased as the temperature is raised.

#### Recovery of protein after dialysis

Measurements of the concentration of protein after dialysis show that the recovery is close at 100% at concentrations of 6  $\mu\text{g/ml}$ , but that this declines steadily with increasing protein concentration, being approx 60% at 30  $\mu\text{g/ml}$  and approx 30% at 80  $\mu\text{g/ml}$ . The lower recovery at high concentrations reflects the aggregation referred to above. There is little effect of omitting dithiothreitol or changing the temperature (from 4 to 20°C) on the level of recovery of protein.

#### c.d. of enzyme after dialysis

The far u.v. c.d. spectrum after dialysis of denatured enzyme is shown in Fig. 2. At an enzyme concentration of 10  $\mu\text{g/ml}$ , there is almost complete regain of native secondary structure provided dithiothreitol is present. In the absence of reducing agent, much less of the native secondary structure

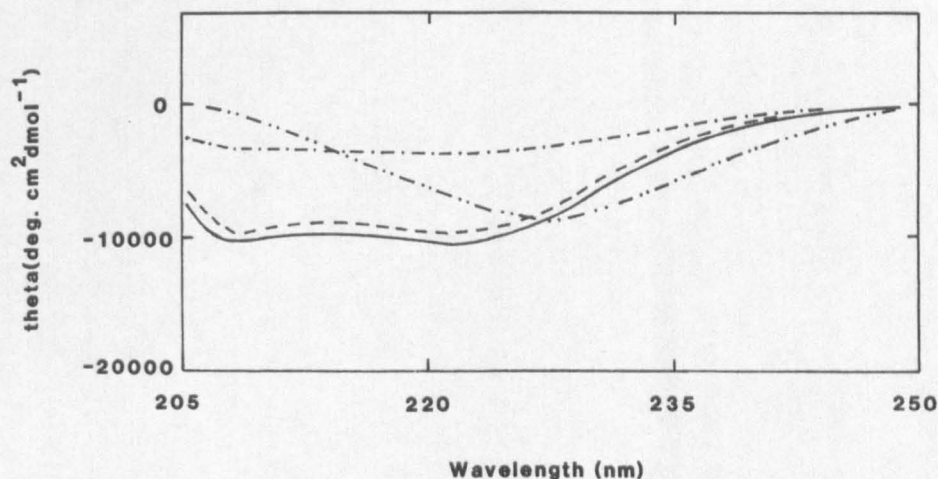


Fig. 2. Far u.v. c.d. spectra of citrate synthase after dialysis. (—), enzyme (10  $\mu\text{g/ml}$ ) not subjected to denaturation; (---), enzyme (10  $\mu\text{g/ml}$ ) denatured and dialysed in the presence of 1 mM dithiothreitol; (-·-·), enzyme (10  $\mu\text{g/ml}$ ) denatured and dialysed in the absence of dithiothreitol; (····) enzyme, (37  $\mu\text{g/ml}$ ) denatured and dialysed in the presence of 1 mM dithiothreitol.



[which consists largely of  $\alpha$ -helix (Remington *et al.*, 1982)] is regained. This would account for the lack of reactivation under these conditions (Fig. 1). At a higher concentration of enzyme (37  $\mu\text{g/ml}$ ) there is a distinct change in the shape of the c.d. spectrum with a single minimum observed at about 225 nm instead of the broad minimum between 210 and 225 nm characteristic of native enzyme (Fig. 2). The c.d. spectrum observed in this case presumably reflects the formation of aggregates which occurs under these conditions. As discussed elsewhere (Adler *et al.*, 1973) the presence of aggregates distorts a c.d. spectrum (mainly by absorption flattening) leading to a red shift and reduction in intensity.

#### DISCUSSION

In this paper we have shown that citrate synthase can be reactivated to a significant extent after denaturation in 6 M GdnHCl, provided that the concentration of the denaturing agent is lowered by dialysis. If the [GdnHCl] is lowered by dilution, there is negligible (<5%) recovery of activity under these conditions (West *et al.*, 1990; Buchner *et al.*, 1991). In this respect citrate synthase behaves in an analogous fashion to another imported mitochondrial protein, fumarase, which can be reactivated by dialysis, rather than by dilution (Kelly and Price, 1991). It is likely that the success of the dialysis procedure arises from the gradual lowering of the concentration of GdnHCl so that correct structural adjustments between intermediates on the folding pathway can occur. By contrast, when the concentration of GdnHCl is lowered suddenly (e.g. by dilution), the enzyme may be prevented from acquiring its native structure by the inability of intermediates to surmount energy barriers at low concentrations of denaturing agent, where exposure of hydrophobic side chains is unfavourable.

Recently, Buchner *et al.* (1991) demonstrated that the addition of bacterial chaperone proteins *groEL* and *groES* could lead to the partial recovery of activity of citrate synthase on dilution of the denatured enzyme, provided ATP was also present. It was concluded that the chaperone proteins acted by inhibiting aggregation reactions which compete with correct protein folding. The extents of regain of activity observed by Buchner *et al.* (1991) are slightly lower than those reported in this paper. It will be of interest to see whether additive effects could be obtained by including chaperone proteins in the dialysis procedure.

The *in vivo* significance of the results reported here remains to be established. It is known that imported mitochondrial proteins are translocated into the organelle in an unfolded (or, at least, non-native) state (Bychkova *et al.*, 1988; Meyer, 1988) and thus after processing must refold and reassemble. The dependence of the reactivation of citrate synthase and other imported enzymes on the refolding conditions implies that special mechanisms must operate in the organelles to ensure that correct folding and assembly of proteins occurs. In view of the results of Buchner *et al.* (1991) and Goloubinoff *et al.* (1989), it is likely that chaperone proteins play an important part in this process. However, the fact that only partial reactivation was observed in these experiments indicates that

a suitable *in vitro* model for this refolding process may still be some way off. The partial success of the dialysis procedure reported in this paper may have useful technological applications in the refolding of denatured proteins which is often required in the recovery of overexpressed recombinant proteins from insoluble inclusion bodies (Marston, 1986).

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## The unfolding and attempted refolding of the bacterial chaperone protein groEL (cpn60)

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The unfolding of the bacterial chaperone protein groEL (cpn60) in solutions of guanidinium chloride (GdnHCl) has been studied. From the results of CD, fluorescence and light scattering, it is clear that major structural transitions in the protein occur over the range 1.0–1.5 M GdnHCl. The ATPase activity of the protein is lost at lower concentrations (0.75 M). After denaturation in concentrations of GdnHCl above 1.5 M, removal of the denaturing agent by dialysis results in very nearly complete regain of secondary structure (as judged by CD), but not the regain of correct tertiary or quaternary structure, or ATPase activity. The product was shown to be very sensitive to proteolysis by thermolysin, unlike the native protein, and not to show enhanced binding of ANS, a characteristic property of the 'molten globule' state of proteins. The results are discussed in relation to current information concerning the assembly of the groEL protein.

### Introduction

The proteins encoded by the *groE* gene of *Escherichia coli* were discovered as critical components for the assembly of large bacteriophages such as lambda and T4 [1,2]. These proteins, groEL, a tetradecamer of subunit  $M_r$  57 200 (also known as cpn60), and groES, a heptamer of subunit  $M_r$  10 300 (also known as cpn10) have been purified [3,4]. They are the subject of a great deal of current research aimed at elucidating (a), the mechanism by which they assist in the correct folding and assembly of other proteins and (b), the relationships between the various groups of such 'chaperone proteins' [5–7]. The mechanism of assembly of the groEL protein and the mitochondrial hsp60 analogue also pose interesting problems [8,9]. In order to examine some aspects of these processes we have examined the unfolding of the groEL protein brought about by guanidinium chloride (GdnHCl), monitoring changes in enzyme activity and in secondary, tertiary and quaternary structure. By removing the denaturing agent, it

should be possible to initiate the refolding process; a technique which has been widely used to explore aspects of the folding pathway of a number of proteins (for reviews, see Refs. 10,11). In the case of the groEL protein, we find that although the secondary structure is efficiently regained after unfolding, the correct tertiary and quaternary structures and enzyme activity are not regained. Some preliminary analysis of the product obtained after removal of the denaturant has been undertaken using the enhancement of ANS fluorescence as a probe for the 'molten globule' state of proteins [12,13], and the susceptibility to proteolysis by thermolysin [14] as a measure of the compactness of the structure.

### Materials and Methods

The groEL protein was purified from *E. coli* over-producing strain DH1pND5 [15] using a procedure modified from that described previously [3,4]. Cells were broken in a French pressure cell. Following negative chromatography on Whatman DE52, ammonium-sulphate fractionation and gel filtration on Sephacryl S-200, the groEL containing fractions (identified by electrophoresis on 15% SDS-polyacrylamide gels) were pooled and dialysed vs. 20 mM Tris-HCl (pH 7.5), containing 5 mM EDTA (buffer A) and applied to a

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Abbreviations: GdnHCl, guanidinium chloride; ANS, 8-anilino-1-naphthalenesulphonate.

Pharmacia Mono-Q column (1 ml) equilibrated with buffer A. The column was eluted with a 0–1 M linear NaCl gradient in buffer A; the groEL protein eluted at 0.45 M NaCl. The pooled groEL containing fractions were dialysed against 50 mM Tris-HCl (pH 7.5), containing 1 mM EDTA and 0.2 M NaCl (buffer B), concentrated by ultrafiltration using Amicon Centriprep 30 cells and 0.5 ml samples applied to a Pharmacia Superose 6 column (24 ml) and eluted with buffer B. The groEL containing fractions, which were more than 99% homogeneous as judged by gel scanning of Coomassie-blue-stained SDS-polyacrylamide gels [16], were pooled and dialysed against 50 mM Tris-HCl (pH 7.5), containing 50% (v/v) glycerol and stored at  $-18^{\circ}\text{C}$ . The characterisation and quantitation of the protein was performed as previously described using a value of 0.285 for the specific absorption coefficient (litre/g per cm) at 280 nm [17].

GdnHCl and urea (ultrapure grade) were purchased from Gibco BRL (Paisley, UK). The concentrations of solutions of GdnHCl and urea were checked by refractive index measurements [18,19]. Thermolysin and ANS (hemimagnesium salt) were purchased from Sigma (Poole, UK).

The unfolding of groEL was studied in the buffer system used by Lissin et al. [9] in their studies of the assembly of the protein, namely 50 mM potassium phosphate, 20 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM dithiothreitol (pH 7.5). Solutions of groEL were incubated for 15 min at  $20^{\circ}\text{C}$  before readings were taken; there was no further significant change on incubation for a further 45 min. In order to remove the denaturing agent, samples were dialysed at

$20^{\circ}\text{C}$  against two changes of 200 volumes of buffer. Measurements of refractive index confirmed that after dialysis the residual GdnHCl concentration was less than 0.01 M.

Fluorescence and light scattering measurements were made at  $20^{\circ}\text{C}$  on a Perkin Elmer LS50 spectrofluorimeter using methods described previously [20]. The changes in  $M_r$  of the protein on addition of GdnHCl were calculated using the published methods [21,22].

The fluorescence of ANS ( $20\ \mu\text{M}$ ) in the presence or absence of protein was measured using excitation and emission wavelengths of 380 nm and 470 nm, respectively.

CD spectra were recorded at  $20^{\circ}\text{C}$  on a JASCO J60C spectropolarimeter; determination of the secondary structure content was undertaken using the Contin procedure at 0.2-nm intervals over the range 240–190 nm [23].

The ATPase activity of groEL was monitored spectrophotometrically at 340 nm by a coupled assay procedure involving pyruvate kinase and lactate dehydrogenase. Concentrations of substrates and coupling enzymes in the buffer described above were: ATP, 0.5 mM; phosphoenolpyruvate, 1.0 mM, NADH, 0.15 mM; pyruvate kinase 25  $\mu\text{g}/\text{ml}$ ; lactate dehydrogenase 8.3  $\mu\text{g}/\text{ml}$  ( $20^{\circ}\text{C}$ ). The amounts of groEL used in the assay were between 20 and 40  $\mu\text{g}$ . Under these conditions the rate of production of ADP was constant for at least 60 min, and was proportional to the amount of groEL added. When the effect of GdnHCl on the ATPase activity was being studied, a 'coupled quench' assay procedure of the type described by Johnson and Price

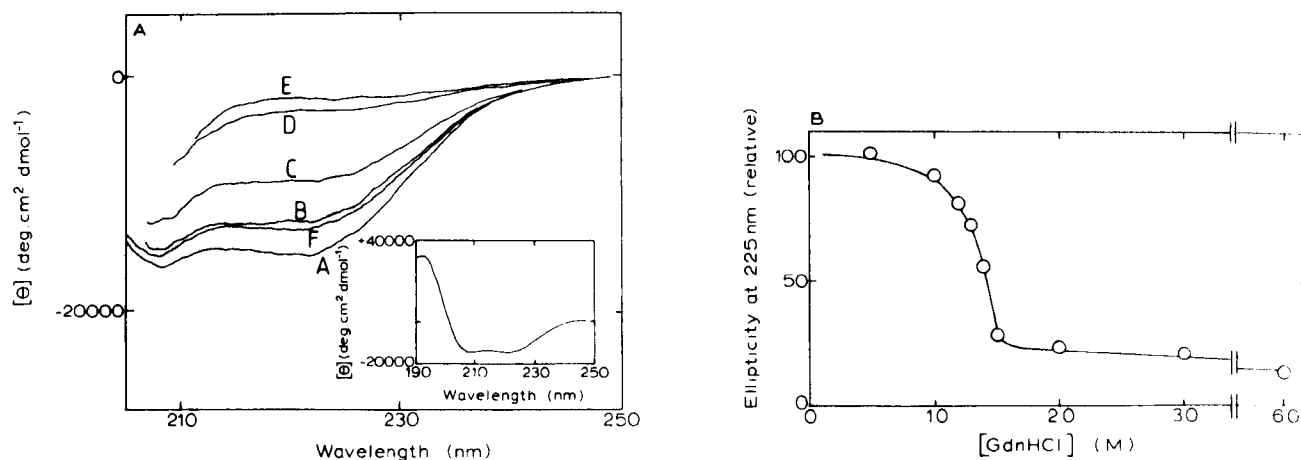


Fig. 1. Changes in the far-UV CD spectrum of groEL in the presence of GdnHCl. Spectra were obtained in 50 mM potassium phosphate, 20 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM dithiothreitol (pH 7.5) at  $20^{\circ}\text{C}$ . (a), In the main figure, the groEL concentration was 50  $\mu\text{g}/\text{ml}$  and spectra were recorded in a cell of pathlength 1 mm. Spectra A, B, C, D and E refer to GdnHCl concentrations of 0, 1.25, 1.4, 2 and 6 M, respectively; spectrum F refers to the sample incubated in 6 M GdnHCl and then dialysed to remove the denaturing agent. The inset shows the spectrum of groEL (0.25 mg/ml) recorded in a cell of pathlength 0.2 mm. This spectrum was used for the estimates of secondary structure described in the text; over the range 250 nm to 205 nm the spectrum was superimposable with spectrum A shown in the main figure. (b), Changes in the ellipticity at 225 nm shown relative to that of groEL in the absence of GdnHCl.

[24] was used, in view of the likely effects of GdnHCl on the coupling enzymes. 0.2 ml aliquots of the reaction mixture (containing groEL and ATP) were added to 0.8 ml samples containing the necessary coupling enzymes and substrates; the very rapid decline in  $A_{340}$  corresponded to the concentration of ADP formed during the ATPase reaction. By taking aliquots at 20 min intervals, the rate of the ATPase reaction could be calculated. Control experiments of the type described previously [24] were undertaken to confirm the validity of the method.

The susceptibility of groEL to thermolysin was studied by incubating groEL (50  $\mu\text{g/ml}$ ) with thermolysin (1–50  $\mu\text{g/ml}$ ) at 20°C for 10 min. The proteinase was then inactivated by addition of EDTA (final concentration 2 mM) [14], and samples analysed by SDS-PAGE [16].

## Results

### Unfolding of groEL by GdnHCl

A number of methods were used to monitor the unfolding of groEL, as outlined below.

**Changes in CD.** The far-UV CD spectra of groEL in the absence of GdnHCl and in the presence of selected concentrations of the denaturing agent are shown in Fig. 1a. In the absence of GdnHCl, the secondary structure content (derived from the spectrum over the range 240–190 nm) corresponds to  $44 \pm 1\%$   $\alpha$ -helix,  $29 \pm 1\%$   $\beta$ -sheet,  $27 \pm 2\%$  remainder. The helix content is somewhat lower than that reported (57%) by Lissin et al. [9]; the amplitude of our spectrum is some 12% lower than that reported by these workers. At least part of the discrepancy could arise from the difficulties in accurate determinations of the concentrations of solutions of groEL [17]. Using the method of Chang et al. [25] to analyse the CD spectrum the contents of  $\alpha$ -helix,  $\beta$ -sheet and remainder are 48%, 37% and 15%, respectively. The method of Siegel et al. [26], which uses data over a more limited range of wavelengths (240–210 nm) gives an estimate of  $58 \pm 2\%$   $\alpha$ -helix.

The changes in the ellipticity at 225 nm on addition of GdnHCl are shown in Fig. 1b. It is clear that there is a sharp change in the range from 1.0 to 1.5 M GdnHCl, with a mid-point in the region of 1.35 M. In some preliminary experiments we have found that at 2°C the mid-point of the transition is shifted to a slightly lower concentration of GdnHCl (1.25 M), supporting the observation of Lissin et al. [9] that groEL is rather less stable at the lower temperature. In experiments using urea as a denaturing agent, the mid-point values were 3.3 M and 3.1 M at 20°C and 2°C, respectively; these values are consistent with those reported (4 M and 3.5 M, respectively) by Lissin et al. [9] as being required for disassembly of the groEL molecule.

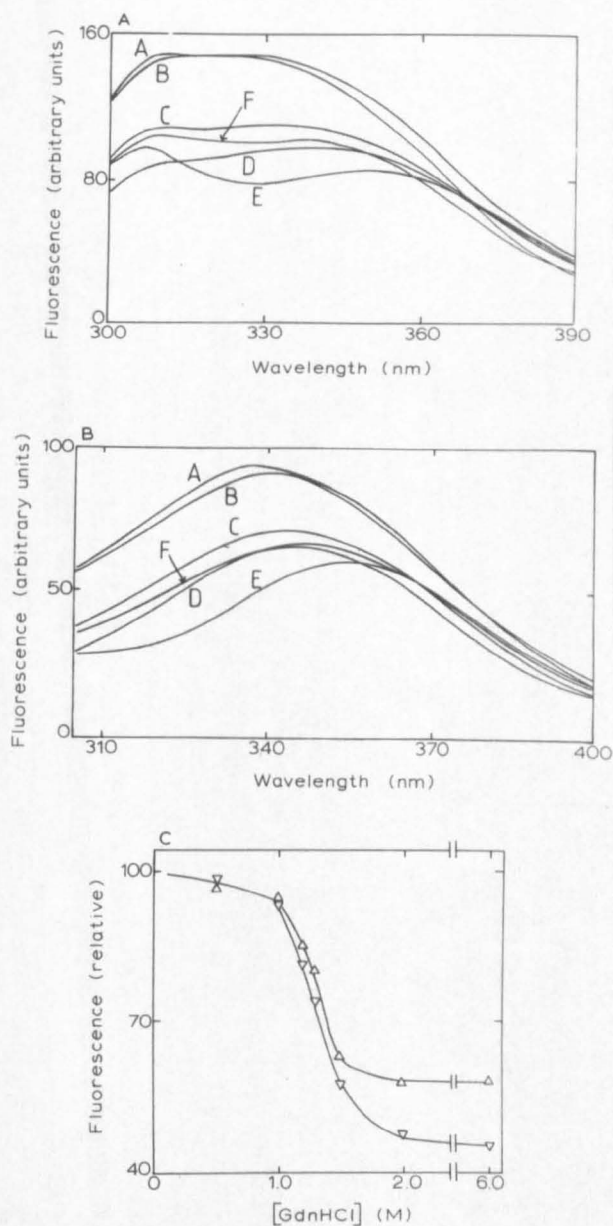


Fig. 2. Changes in the fluorescence of groEL in the presence of GdnHCl. Spectra were recorded at a groEL concentration of 5  $\mu\text{g/ml}$  in 50 mM potassium phosphate, 20 mM potassium acetate, 1 mM magnesium acetate, 0.1 mM dithiothreitol (pH 7.5) at 20°C. Spectra A, B, C, D and E refer to GdnHCl concentrations of 0, 1.0, 1.3, 1.5 and 6.0 M, respectively. Spectrum F refers to the sample incubated in 6 M GdnHCl and then dialysed to remove the denaturing agent. (a) and (b) refer to excitation at 280 nm and 290 nm, respectively; (c) shows the relative changes in fluorescence at 315 nm when excited at 280 nm ( $\Delta$ ) or 290 nm ( $\nabla$ ).

**Changes in fluorescence.** The fluorescence emission spectra of groEL in the presence of varying concentrations of GdnHCl are shown in Fig. 2a and 2b, which refer to excitation at 280 and 290 nm, respectively. In each case, it is clear that major changes occur in the spectrum between 1.0 and 1.5 M GdnHCl; Fig. 2c shows these effects in terms of the changes in intensity.

at 315 nm. There is a shift in the wavelength maximum as the protein unfolds; this is easier to appreciate in Fig. 2b, where tryptophan fluorescence is selectively excited, than in Fig. 2a where tyrosine and tryptophan both contribute to the emission [17]. In the absence of GdnHCl, the wavelength of maximum emission is 335 nm; this is shifted to 355 nm, the value characteristic of solvent exposed tryptophan, in 6 M GdnHCl.

**Changes in light scattering.** The changes in  $M_r$  of the groEL protein at different concentrations of GdnHCl were monitored by changes in the intensity of light scattering at 360 nm. The results (Fig. 3) show that there is a marked loss of quaternary structure between 1.0 and 1.5 M GdnHCl.

**Changes in ATPase activity.** The groEL protein possesses weak ATPase activity [3] and it has been demonstrated that ATP hydrolysis is a requirement for the assistance of groEL and groES in protein folding *in vitro* [27,28]. In the absence of GdnHCl, the ATPase activity corresponds to a  $k_{cat}$  (expressed per subunit of  $M_r$  57 200) of  $0.060 \pm 0.005 \text{ s}^{-1}$ , as measured in our coupled assay procedure. This value is similar to that measured under slightly different conditions using measurements of the release of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ; thus Hendrix [3] reported a value of  $0.057 \text{ s}^{-1}$  and Viitanen et al. [29] quoted a value of  $0.075 \text{ s}^{-1}$ . Fig. 4 shows the loss of ATPase activity; essentially all activity is lost by 0.75 M GdnHCl.

**Comparison of the various techniques.** The various methods for monitoring the unfolding of the groEL protein are compared in Fig. 4, where the data are normalised to show the changes as a percentage of the maximum change observed. The various measures of secondary, tertiary and quaternary structure all show major changes between 1.0 and 1.5 M GdnHCl. The steepness of the changes in this region and the lack of any discernible plateau suggest that unfolding of the

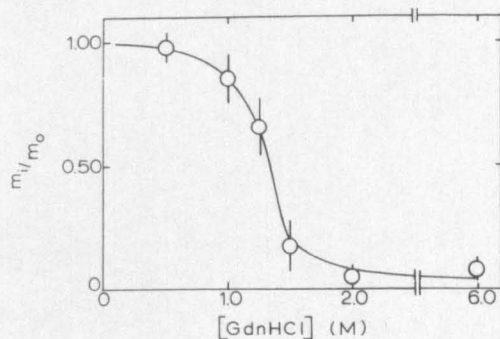


Fig. 3. Changes in  $M_r$  of groEL in the presence of GdnHCl. The changes in  $M_r$  were determined by light scattering as described in the text. The ordinate represents the ratio of  $M_r$  in the presence of GdnHCl to that in the absence of GdnHCl. The concentration of groEL was  $50 \mu\text{g/ml}$  in 50 mM potassium phosphate, 20 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM dithiothreitol (pH 7.5) at  $20^\circ\text{C}$ . The experimental points represent the means of 4 determinations.

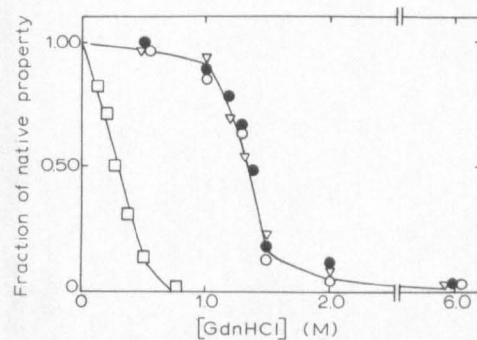


Fig. 4. Changes in the ATPase activity and structural parameters of groEL in the presence of GdnHCl. The data are normalised to show the difference between protein in the absence and in the presence of 6 M GdnHCl. (□) changes in ATPase activity; (●) changes in ellipticity at 225 nm; (○) changes in  $M_r$  from light scattering; (▽) changes in fluorescence at 315 nm (excited at 290 nm). The concentration of groEL was  $50 \mu\text{g/ml}$  for the structural measurements and  $40 \mu\text{g/ml}$  for the activity measurements. The buffer was 50 mM potassium phosphate, 20 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM dithiothreitol (pH 7.5) at  $20^\circ\text{C}$ . For the sake of clarity, the changes in fluorescence at 315 nm (excited at 280 nm) are not shown; these run broadly in parallel with those excited at 290 nm (see Fig. 2c).

tetradecamer is a highly cooperative process. By contrast, the enzymatic activity of the groEL protein is lost at lower concentrations of GdnHCl, where there is little evidence for significant overall structural changes. It is likely that the more pronounced loss of enzyme activity reflects the greater sensitivity of the active site to structural perturbations as has been observed in other systems [30,31]; however, an alternative explanation for the loss of activity could be that the denaturant binds to important charged side chains at the active site.

#### The attempted refolding of groEL

Following denaturation by incubation with GdnHCl, refolding of groEL was attempted by dialysing the protein against buffer as outlined in Materials and Methods; during this process, 0.1 mM dithiothreitol was added in order to prevent oxidation of the cysteine side chains in the protein. When the initial concentration of GdnHCl was 1.0 M or below, there was essentially complete restoration of the secondary, tertiary and quaternary structure of the groEL protein, as revealed by CD, fluorescence and light scattering, respectively. In addition the ATPase activity of the protein was restored (Fig. 5). However, when the initial concentration of GdnHCl was raised progressively above 1.0 M, there was a decreasing extent of regain of ATPase activity (Fig. 5); this was correlated with a progressive loss of ability to regain correct tertiary structure as monitored by the wavelength of maximum emission when excited at 290 nm. As shown in Fig. 2b, when the initial concentration of GdnHCl was 6.0 M

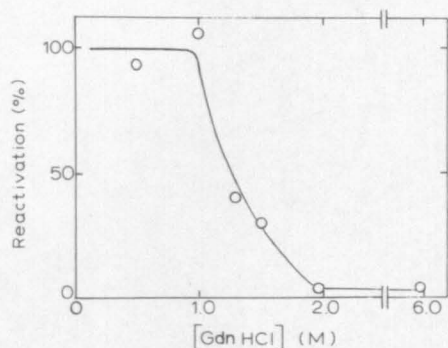


Fig. 5. Reactivation of groEL after incubation in GdnHCl. GroEL (50  $\mu\text{g/ml}$ ) was incubated with the stated concentrations of GdnHCl for 15 min at 20°C; the denaturing agent was then removed by dialysis. The degree of reactivation is expressed relative to a control sample incubated in the absence of GdnHCl. Experiments were performed in 50 mM potassium phosphate, 20 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM dithiothreitol (pH 7.5).

(corresponding to complete unfolding of the protein), the wavelength of maximum emission after dialysis was 345 nm, intermediate between that of the native (335 nm) and unfolded (355 nm) protein. The measurements of quaternary structure by light scattering revealed that under these conditions the native quaternary structure had not been regained, since in most experiments the intensity of light scattering was only some 40–50% of the control sample which had been incubated in the absence of GdnHCl and subsequently

dialysed. (It should be noted that on a few occasions, the solutions became slightly turbid under these conditions, suggesting that aggregate formation had occurred). However, irrespective of the initial concentration of GdnHCl, the regain of secondary structure as revealed by CD is almost complete; Fig. 1a shows the data for the sample with an initial concentration of GdnHCl of 6.0 M; in this case, approx. 90% of the ellipticity at 225 nm is restored. In some preliminary experiments, it was found that when the dialysis was performed at 2°C, rather than 20°C, the regain of secondary structure was less efficient (57% of the ellipticity at 225 nm was restored).

Further characterisation of the product obtained after dialysis was undertaken by studies of ANS binding and of susceptibility to proteolysis by thermolysin. The fluorescence of ANS (20  $\mu\text{M}$ ) in the presence of 50  $\mu\text{g/ml}$  groEL was enhanced approx. 2.2-fold, compared with free ANS. This small enhancement declined further when GdnHCl was included over the range between 0 and 6 M (approx. 1.5-fold at 6 M), consistent with the general observation, [12,13] that ANS does not bind to any great extent to either the native or denatured states of proteins. The product obtained after dialysis gave only a 2-fold enhancement (compared with free ANS), suggesting that it does not possess a major characteristic of the 'molten globule' state (i.e., enhanced binding of ANS) [12,13]. By contrast, in preliminary experiments which have been undertaken with mitochondrial malate dehydrogenase and

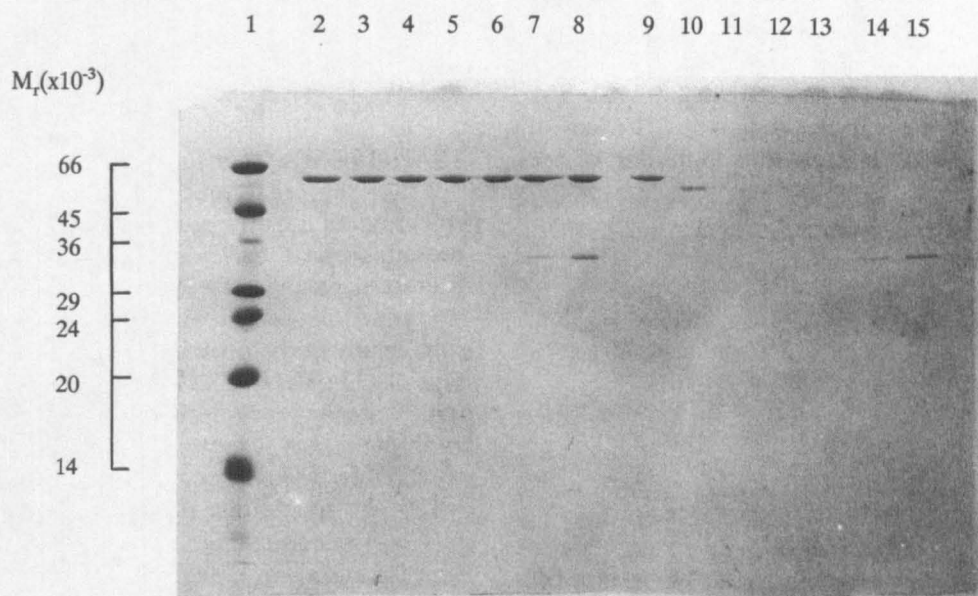


Fig. 6. Susceptibility of groEL to proteolysis by thermolysin. GroEL (50  $\mu\text{g/ml}$ ) was incubated with thermolysin for 10 min at 20°C in 50 mM potassium phosphate, 20 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM dithiothreitol (pH 7.5). EDTA was then added to a final concentration of 2 mM and samples were analysed by SDS-PAGE on 15% acrylamide gels. Lanes 1, 2–8, and 9–15 contain  $M_r$  markers, native groEL, and groEL which has been denatured and dialysed, respectively. The concentrations of thermolysin ( $\mu\text{g/ml}$ ) added were: lanes 2 and 9, 0; lanes 3 and 10, 1; lanes 4 and 11, 2; lanes 5 and 12, 5; lanes 6 and 13, 10; lanes 7 and 14, 20; lanes 8 and 15, 50. The band at  $M_r$  33000 in lanes 7, 8, 14 and 15 corresponds to thermolysin.

citrate synthase, both of which can be only partially (20–30%) reactivated by dialysis of the denatured protein, significant enhancement of ANS binding was observed. The enhancement of ANS fluorescence was 9.9- and 4.6-fold, respectively, compared with free ANS, whereas the native or denatured proteins gave enhancements in the range 1.3- to 2.1-fold in each case.

Thermolysin was chosen as the proteinase with which to study the compactness of the structure of the product obtained by dialysis because (a), it is readily inactivated by addition of EDTA, thereby preventing artefactual proteolysis during preparation of samples for SDS-PAGE and (b), the preference for large hydrophobic side-chains valine or isoleucine which occurs only rarely on the surfaces of native proteins usually ensures that there is effective discrimination between native and non-native structures [14]. As shown in Fig. 6 (lanes 2 to 8), native groEL is resistant to thermolysin (2–100% (w/w)) as judged by SDS-PAGE. By contrast, the product obtained by dialysis of the denatured protein is very susceptible to proteolysis. After 10 min incubation with 2% (w/w) thermolysin only a small amount of material corresponding to  $M_r$  54 000 could be observed (Fig. 6, lane 10); the intensity of this band corresponds to 28% of that observed for the intact polypeptide chain ( $M_r$  57 000) in the absence of thermolysin (Fig. 6, lane 9). No distinct bands corresponding to digestion products with  $M_r$  greater than 14 000 could be observed when larger amounts of proteinase were added (Fig. 6, lanes 11–15).

## Discussion

The results reported in this paper show that GdnHCl causes major structural changes in groEL when the concentration of denaturing agent is raised above about 1.0 M. As shown in Fig. 4, the changes in secondary, tertiary and quaternary structure run in parallel. It should be noted that because the unfolding process cannot (under these conditions) be considered reversible, the changes in the various properties studied do not represent the attainment of a true equilibrium between native and unfolded states of the protein. Instead the protein, especially in the 'transition region' between 1.0 and 1.5 M GdnHCl, should be regarded as rapidly attaining a metastable state, which we have observed to be stable over a period of 1 h. The steep loss of structure over a relatively narrow range of GdnHCl concentrations, points to a cooperative unfolding of the tetradecameric protein. This behaviour is to be contrasted with, for example, that of the hexameric glutamate dehydrogenase which dissociates and unfolds in two stages via a trimeric intermediate as the GdnHCl concentration is increased [20].

Attempts to refold correctly the denatured groEL by the dialysis technique have proved unsuccessful when

the initial GdnHCl concentration is above the level at which major structural alterations occur on the time scale of these experiments. In this respect, the behaviour of groEL is analogous to glutamate dehydrogenase [20], aspartate aminotransferase [32,33], citrate synthase [34] and fumarase [31]. In at least the last two cases, dialysis of the unfolded protein has given substantially higher yields of activity than rapid dilution of the denaturing agent [31,35].

The inability of the groEL protein to refold and reassemble correctly after denaturation poses interesting questions concerning the assembly of the protein *in vivo*. Lissin et al. [9] concluded that the correct assembly of the groEL tetradecamer after unfolding in 4 M urea required the intervention of groES and the hydrolysis of ATP, and postulated the idea of 'self-chaperoning' of the assembly process. Cheng et al. [8] in a study of the yeast mutant *mif4*, concluded that assembly *in vivo* of the homologous hsp60 complex in mitochondria required the presence of pre-existing functional complex. Our studies show that in the case of groEL, secondary structure can be regained with high efficiency on refolding. The results obtained with the smallest amount of thermolysin used suggest that a folded structure involving most (54 kDa out of 57 kDa) of the polypeptide chain has been formed. However, this product still has a relatively open structure compared with native groEL, since it is degraded by larger amounts of proteinase. As shown by our other results, formation of the correct tertiary and quaternary structures and appearance of ATPase activity are unable to proceed. It remains a task for future work to characterise this folded state in more detail and to explore the role (if any) played by groES and/or ATP hydrolysis in the later steps involved in the acquisition of the correct tertiary and quaternary structures of the groEL protein.

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# Unfolding and refolding of the NAD<sup>+</sup>-dependent isocitrate dehydrogenase from yeast

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*The unfolding of the NAD<sup>+</sup>-dependent isocitrate dehydrogenase from yeast in guanidinium chloride (GdnHCl) has been monitored by changes in c.d. and fluorescence. Major structural changes occur over the range of GdnHCl concentrations from 0.5 to 1.5 M, although loss of catalytic activity is complete at 0.3 M. After incubation in GdnHCl, activity can be regained on dilution; however, the extent of this regain is dependent on the initial concentration of GdnHCl and is very small at a concentration of 2 M or above. Under these conditions there is only limited regain of the secondary and tertiary structure of the enzyme. Considerably more structure and activity can be regained if the concentration of GdnHCl is lowered by dialysis. The implications of these results for the folding and assembly of the enzyme are discussed.*

**Keywords:** Isocitrate dehydrogenase; denaturation; unfolding; refolding; circular dichroism; fluorescence

## Introduction

Isocitrate dehydrogenase catalyses the oxidative decarboxylation of isocitrate to 2-oxoglutarate. Two forms of the enzyme occur in eukaryotes<sup>1</sup>. The NAD<sup>+</sup>-dependent enzyme (EC 1.1.1.41) is confined to the mitochondrion and catalyses a step in the tricarboxylic acid cycle. The NADP<sup>+</sup>-dependent enzyme (EC 1.1.1.42) occurs predominantly in the cytosol; its function is less well established<sup>1</sup>.

The NAD<sup>+</sup>-dependent enzyme has been purified from a number of sources including yeast<sup>2,3</sup> and pig heart<sup>4,5</sup>. In both cases the enzyme appears to consist of eight subunits each of  $M_r$  about 40 000, although a recent report<sup>6</sup> suggests that the pig heart enzyme may contain three types of subunit all of similar  $M_r$ , but separable by isoelectric focusing. The NAD<sup>+</sup>-dependent enzymes show complex kinetic behaviour<sup>1,6</sup>; in the case of the yeast enzyme this is manifested as a sigmoidal dependence of rate on isocitrate concentration and activation by AMP<sup>3,7</sup>. By contrast the NADP<sup>+</sup>-dependent enzymes do not, in general, show regulatory properties<sup>1,5,8,9</sup>.

As part of our investigations of the folding and assembly of imported mitochondrial enzymes<sup>10-13</sup>, we have undertaken a study of the unfolding of the NAD<sup>+</sup>-dependent isocitrate dehydrogenase from yeast by guanidinium chloride (GdnHCl) and the subsequent refolding of the enzyme on removal of the denaturing agent by dilution or dialysis. The results show that whereas the dialysis procedure leads to a substantial degree of reactivation, the dilution procedure is much less efficient. This situation has been observed with other mitochondrial enzymes, e.g. fumarase<sup>13</sup> and citrate synthase<sup>14</sup> and may point to a general problem concerning the folding and assembly of this group of enzymes *in vivo*.

## Experimental

NAD<sup>+</sup>-dependent isocitrate dehydrogenase from yeast was purchased as a solution in 50% (v/v) glycerol from Calbiochem, Nottingham, UK. Analysis of the preparation by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis on 12% acrylamide gels<sup>15</sup> showed that approximately 60% of the Coomassie Blue staining material migrated with the mobility expected for NAD<sup>+</sup>-dependent isocitrate dehydrogenase (i.e. corresponding to  $M_r$  38 000 ± 2000). The major contaminant, amounting to some 35% of the total staining material, had a mobility corresponding to a subunit  $M_r$  of 52 000 ± 3000. Further purification of the commercial sample was undertaken by ion-exchange chromatography on a monoQ (HR 5/5) column equilibrated with 40 mM Tris-HCl, pH 7.6, containing 4 mM MgCl<sub>2</sub>. A gradient of 0-1 M NaCl in this buffer was applied to the column. Isocitrate dehydrogenase was eluted as a sharp, symmetrical peak at a concentration of 0.17 ± 0.02 M NaCl; the major contaminant was eluted at a concentration of 0.32 ± 0.02 M. The isocitrate dehydrogenase prepared in this way was shown to be at least 95% homogeneous by SDS-polyacrylamide gel electrophoresis<sup>15</sup>. For subsequent work, the NaCl present in the preparations was removed by dialysis against 40 mM Tris-HCl, pH 7.6, containing 4 mM MgCl<sub>2</sub>. Concentrations of enzyme were generally estimated spectrophotometrically using a value of 0.69 for the  $A_{280}$  of 1 mg/ml solution in a 1 cm pathlength cuvette<sup>3</sup>. At low concentrations of protein, a more sensitive Coomassie Blue binding assay<sup>16</sup> was also used; control experiments showed that this method gave values within 10% of those determined by measurements of  $A_{280}$ .

Isocitrate dehydrogenase activity was assayed as described by Kuehn *et al.*<sup>7</sup>, except that the temperature

was 25°C rather than 30°C. The concentrations of substrates etc. were: DL-isocitrate, 0.39 mM; NAD<sup>+</sup>, 0.34 mM; AMP, 1 mM; dithiothreitol, 3 mM in 40 mM Tris-HCl, pH 7.6, containing 4 mM MgCl<sub>2</sub>. At 25°C, the specific activity of the purified preparation was 16 units (i.e.  $\mu\text{mol}$  2-oxoglutarate formed per min) per mg protein. This was shown to correspond to 21.5 units/mg at 30°C and can be compared with the value of 26.2 units/mg reported under the latter conditions<sup>7</sup>. By substituting NADP<sup>+</sup> for NAD<sup>+</sup> in the assay it was shown that the NADP<sup>+</sup>-dependent enzyme was not present in the preparation (less than 0.1% of the activity of the NAD<sup>+</sup>-dependent enzyme).

GdnHCl (Ultrapure grade) was obtained from Gibco-BRL, Paisley, Scotland, UK. The concentrations of solutions were checked by refractive index measurements<sup>17</sup>.

C.d. measurements were made at 25°C on a Jasco J-600 spectropolarimeter. Far u.v. spectra were recorded at protein concentrations in the range 20–150  $\mu\text{g}/\text{ml}$  using cell pathlengths in the range 0.02 to 0.1 cm. Analysis of the spectra in terms of the secondary structure content was performed by applying the CONTIN procedure<sup>18</sup> at 0.2 nm intervals over the range 190 to 240 nm.

Fluorescence spectra were recorded at 25°C on a Perkin-Elmer LS50 spectrofluorimeter. Corrections for Raman scattering were made using appropriate solutions containing no protein. The fluorescence of 8-anilino-1-naphthalenesulphonate (ANS) was monitored at 470 nm, with excitation at 380 nm.

Samples of isocitrate dehydrogenase were incubated with GdnHCl in 40 mM Tris-HCl, pH 7.6, containing 4 mM MgCl<sub>2</sub> and 0.1 mM dithiothreitol, for 15 min at 25°C, before measurement of spectral properties or catalytic activity (in an assay solution containing the same concentration of GdnHCl as in the original incubation). There was no significant change in these properties when incubation was continued for a further 30 min, although very slow further changes were observed when samples containing between 0.5 and 3 M GdnHCl were incubated over a further period of 24 h. In experiments where the refolding of enzyme was studied the samples were then either diluted (at 25°C) with 40 mM Tris-HCl, pH 7.6, containing 4 mM MgCl<sub>2</sub> and 0.1 mM dithiothreitol, so as to lower the concentration of GdnHCl to  $\leq 0.05$  M, or dialysed at 25°C over a 5 h period against two changes of this buffer. The latter procedure was shown to lower the residual concentration of GdnHCl to  $\leq 0.02$  M.

## Results

### Spectral properties of native isocitrate dehydrogenase

C.d. The far u.v. c.d. spectrum of isocitrate dehydrogenase is shown in Figure 1a. Analysis of the spectrum over the range 190 to 240 nm by the CONTIN procedure<sup>18</sup> gives the following estimates of secondary structure:  $\alpha$ -helix, 36%;  $\beta$ -sheet, 29%; remainder, 35%. The method of Siegel *et al.*<sup>19</sup> which analyses data over a more restricted range of wavelengths (210 to 240 nm) gives an  $\alpha$ -helix content of 43%.

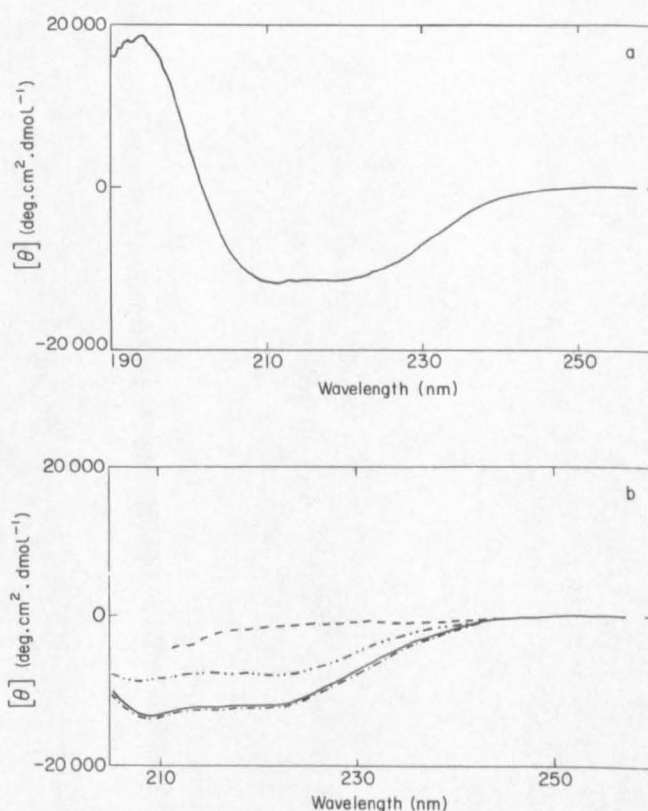
Although there are at present no X-ray structural data with which to compare these estimates, it is of interest to note that other NAD<sup>+</sup>-linked dehydrogenase (with which isocitrate dehydrogenase presumably shares at

least some elements of supersecondary structure) have  $\alpha$ -helix and  $\beta$ -sheet contents in this range. Thus lactate, malate and glyceraldehyde-3-phosphate dehydrogenases have 40%, 49% and 36%  $\alpha$ -helix respectively and 23%, 25% and 40%  $\beta$ -sheet respectively<sup>20,21,22</sup>.

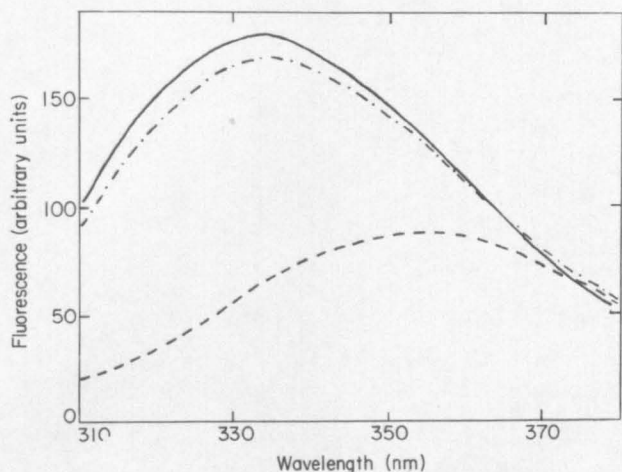
Fluorescence. When excited at 290 nm, isocitrate dehydrogenase exhibits an emission maximum at 332 nm (Figure 2), showing that the three tryptophan side chains per subunit<sup>3</sup> are on average in an environment which is moderately exposed to solvent<sup>23</sup>.

### Unfolding of isocitrate dehydrogenase by GdnHCl

As shown in Figures 1b and 2, incubation in 6 M GdnHCl causes complete loss of the secondary and tertiary structure of isocitrate dehydrogenase. The far u.v. c.d. spectrum (Figure 1b) is characteristic of a random coil with the ellipticity at 225 nm reduced to less than 10% of its value in the absence of GdnHCl. The fluorescence emission maximum is shifted to 355 nm



**Figure 1** Far u.v. c.d. spectra of isocitrate dehydrogenase. Spectra were recorded in 40 mM Tris-HCl, pH 7.6, containing 4 mM MgCl<sub>2</sub> and 0.1 mM dithiothreitol. Molar ellipticities were calculated assuming a value of 108 for the mean residue weight<sup>3</sup>. (a) Spectrum of enzyme (150  $\mu\text{g}/\text{ml}$ ) recorded in a cell of pathlength 0.02 cm. (b) Spectra of enzyme samples recorded at lower concentrations in a cell of pathlength 0.1 cm. It should be noted that the spectra of enzyme recorded at 30  $\mu\text{g}/\text{ml}$  or 50  $\mu\text{g}/\text{ml}$  in the absence of GdnHCl (—) are superimposable with that shown in (a) down to 200 nm; below this wavelength, the spectra show considerable noise. (---) is the spectrum of enzyme (50  $\mu\text{g}/\text{ml}$ ) incubated in the presence of 6 M GdnHCl; below 210 nm the spectrum shows excessive noise. (- · - · -) is the spectrum of enzyme (50  $\mu\text{g}/\text{ml}$ ) incubated in the presence of 6 M GdnHCl and subsequently dialysed to remove the denaturing agent. (- · · · -) is the spectrum of enzyme incubated in the presence of 3 M GdnHCl and then diluted 60-fold to give a final enzyme concentration of 30  $\mu\text{g}/\text{ml}$



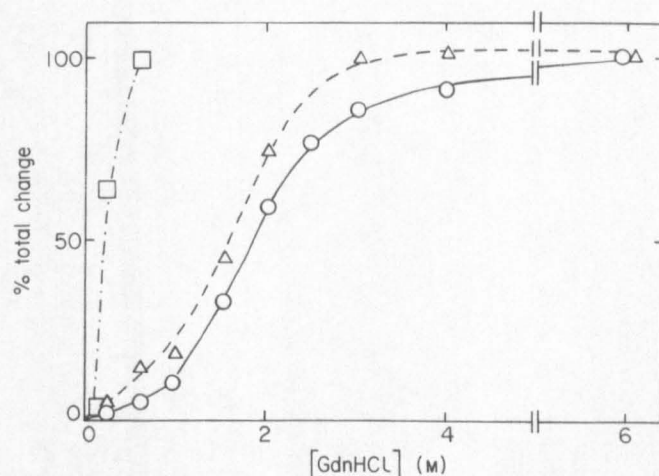
**Figure 2** Fluorescence spectra of isocitrate dehydrogenase recorded at 25°C in 40 mM Tris-HCl, pH 7.6, containing 4 mM MgCl<sub>2</sub> and 0.1 mM dithiothreitol. The enzyme concentration was 50 µg/ml in each case, and the excitation wavelength 290 nm. (—), (---) and (-·-·-) refer to native enzyme, enzyme incubated in the presence of 6 M GdnHCl, and enzyme incubated in the presence of 6 M GdnHCl and subsequently dialysed respectively

(Figure 2), a value characteristic of fully exposed tryptophan side chains<sup>23</sup>. The changes in secondary structure (ellipticity at 225 nm) and tertiary structure (fluorescence at 335 nm) as a function of GdnHCl concentration are shown in Figure 3. The unfolding curves obtained at enzyme concentrations of 50 µg/ml and 30 µg/ml were indistinguishable. The two types of structural changes occur roughly in parallel with the major changes observed in the range of GdnHCl concentrations from 0.5 to 1.5 M. As has been found elsewhere (for example, in the case of fumarase<sup>13</sup>) the loss of tertiary structure runs slightly ahead of the loss of secondary structure, but there is no evidence from these data for the existence of significantly populated intermediates, such as the 'molten globule'<sup>24</sup> which possesses secondary structure but little stable tertiary structure. This conclusion is supported by the results of studies of the binding of ANS, which has been regarded as a probe for the 'molten globule' state<sup>24</sup>. The enhancement of fluorescence of 20 µM ANS in the presence of 50 µg/ml isocitrate dehydrogenase was 6.8-fold compared with free ANS. As the concentration of GdnHCl was increased over the range from 0 to 4 M, no evidence was obtained for increased binding of ANS. Rather, there was a steady decline in the enhancement of fluorescence reaching a value of 1.7-fold at a GdnHCl concentration of 4 M.

Figure 3 also shows that loss of activity of isocitrate dehydrogenase occurs at low concentrations of GdnHCl; thus no activity remains at 0.3 M GdnHCl, despite the fact that no major structural changes have occurred at this point. Although this behaviour probably reflects the greater sensitivity of the active site to structural perturbations compared with the rest of the molecule<sup>25</sup>, it is also possible that the effects may arise from binding of the denaturant to side chains at the active site.

#### Refolding of isocitrate dehydrogenase after incubation in GdnHCl

**Dilution procedure.** The activity of the enzyme was monitored over a period of 240 min after dilution of the

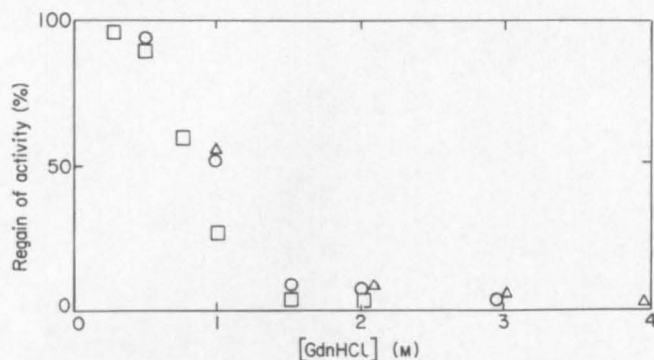


**Figure 3** Unfolding of isocitrate dehydrogenase by GdnHCl. (□), (○) and (Δ) refer to the losses in activity, ellipticity at 225 nm and fluorescence at 335 nm respectively. In each case the protein concentration was 50 µg/ml and the changes are expressed relative to the total change occurring between 0 and 6 M GdnHCl

solution to lower the concentration of GdnHCl to  $\leq 0.05$  M. Although in each experiment a small (<20%) increase was generally observed between the activity of the first sample (taken after 1 min) and that of the sample taken after 60 min, there was no systematic change in activity over the subsequent 180 min. Figure 4 shows the regain of activity after 120 min, in each case expressed relative to a control sample from which GdnHCl was omitted in the initial incubation step. From these data it is clear that the extent of regain of activity is markedly dependent on the concentration of GdnHCl employed in the initial incubation. The ability to regain activity on dilution was essentially lost over the range from 0.5 to 2 M GdnHCl, where major structural transitions occur in the protein. At concentrations of GdnHCl of 4 M and above, no activity was regained over a 240 min period or even after a subsequent 20 h incubation.

The regain of secondary and tertiary structure on dilution was monitored by c.d. and fluorescence measurements respectively, at a final enzyme concentration (after dilution) of 30 µg/ml. At an initial GdnHCl concentration of 0.5 M, the regains of enzyme activity, ellipticity at 225 nm, and fluorescence at 335 nm on dilution were 95%, 94% and 97% respectively. As the initial concentration of GdnHCl was raised, the regain of structure was less complete. Thus when the initial concentration of GdnHCl was 3 M, the regain of activity was <2%, and the regain of ellipticity at 225 nm was 64% (Figure 1b). The regain of fluorescence at 335 nm was 65%, but the emission maximum (337 nm) was significantly different from that of native enzyme (332 nm) indicating that the tryptophan side chains are in different environments from those in the native enzyme. At intermediate initial concentrations of GdnHCl, the regains of activity and structure were at intermediate levels; e.g. at a concentration of 1 M, the regains of activity, ellipticity and fluorescence on dilution were 50%, 76% and 82% respectively.

As is also shown in Figure 4, the extent of regain of activity is somewhat higher as the concentration of enzyme is raised. This is similar to the behaviour of some other multi-subunit enzymes (e.g. yeast phospho-



**Figure 4** Regain of activity of isocitrate dehydrogenase after denaturation by GdnHCl upon subsequent dilution. The activities shown are those measured 120 min after dilution, and in each case are expressed relative to a control sample incubated in the absence of GdnHCl before dilution. (□), (Δ) and (○) refer to enzyme concentrations of 4, 16 and 30 μg/ml after dilution

glycerate mutase<sup>26</sup>) during refolding and presumably reflects the tendency at higher concentrations of proteins for associative steps involved in the regain of catalytic activity to be favoured relative to (lower kinetic order) deactivation processes. However, at the higher concentrations of proteins there was no regain of activity when the initial concentration of GdnHCl was  $\geq 4$  M.

**Dialysis procedure.** When isocitrate dehydrogenase was incubated in the presence of 6 M GdnHCl for 15 min at 25°C and the sample then dialysed to remove GdnHCl, considerable regain of activity occurred. At an initial enzyme concentration of 50 μg/ml, 75% activity was regained relative to a control sample incubated in the absence of GdnHCl. As shown in *Figures 1b* and *2*, the secondary and tertiary structure of the native enzyme were essentially restored under these conditions. At a lower concentration of enzyme (30 μg/ml) the extent of regain of activity was lower (40%) although the secondary and tertiary structures were regained to a similar extent to those occurring at 50 μg/ml. Presumably the lower regain of catalytic activity reflects the diminished occurrence of required associative steps in the refolding pathway.

## Discussion

In this paper, we have shown that the ability of NAD<sup>+</sup>-dependent isocitrate dehydrogenase to refold after incubation in GdnHCl depends on the experimental conditions employed. If the concentration of the denaturing agent is lowered rapidly (by dilution) full activity can only be regained provided the GdnHCl concentration is below that at which major structural changes are observable by c.d. or fluorescence (about 0.5 M). As the concentration of GdnHCl is raised above this value, the extent of regain of activity decreases and is very small indeed above 2 M GdnHCl. This low level of regain of activity is accompanied by the inefficient regain of native secondary and tertiary structure. It should be noted that the regain of activity occurs rapidly (largely within 1 min after dilution); the extent of this regain therefore reflects the proportion of the molecules which can undergo rapid small structural changes to

regenerate the native structure and which have not suffered major, irreversible, structural transitions. This correlation between lack of reactivation on dilution and major structural changes has been noted in earlier studies on a number of mitochondrial enzymes, e.g. glutamate dehydrogenase<sup>10</sup>, aspartate aminotransferase<sup>11</sup>, citrate synthase<sup>12</sup> and fumarase<sup>13</sup>.

When the concentration of the denaturing agent is lowered gradually (by dialysis), considerable activity can be regained even when the initial concentration of GdnHCl is 6 M. The success of the dialysis procedure relative to the dilution procedure has been observed in the cases of fumarase and citrate synthase<sup>13,14</sup> and has been taken to reflect the ability of correct structural adjustments between intermediates on the folding pathway to occur. By contrast, when the concentration of GdnHCl is lowered suddenly by dilution the enzyme may be prevented from acquiring its native structure by the inability of intermediates to surmount energy barriers at low concentrations of denaturing agent where the exposure of hydrophobic side chains is unfavourable. The results from the spectroscopic studies show that neither the correct secondary nor tertiary structure can be regained efficiently, in contrast to the results obtained using the dialysis procedure. In both procedures a tendency for increased reactivation was noted as the protein concentration was raised, pointing to the importance of associative steps in the formation of active species; this has been observed in the refolding of a number of other multi-subunit enzymes<sup>27</sup>.

The vast majority of mitochondrial proteins are encoded by nuclear genes and are imported as precursors in an unfolded (or, at least, non-native) state<sup>28,29</sup>. After processing, the imported proteins must fold and assemble to generate the mature active species. The dependence of the reactivation of NAD<sup>+</sup>-dependent isocitrate dehydrogenase on the refolding conditions employed suggests that special mechanisms must operate *in vivo* to ensure that correct folding occurs. There is now a wealth of evidence<sup>30,31</sup> to indicate that molecular chaperones as well as other proteins play a crucial role in these processes. It will be of interest to perform experiments *in vitro* to study the effects of chaperone proteins such as groEL (cpn 60) and groES (cpn 10) on the refolding of isocitrate dehydrogenase. In the case of citrate synthase, these proteins have been shown to increase the regain of activity after denaturation by suppressing the formation of aggregates.<sup>32</sup>

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