

7.1.1
30.1.1

The Refolding of Riboflavin Binding Protein

David Andrew M^cClelland

**A thesis submitted for the degree of
Doctor of Philosophy**

**Department of Biological and Molecular Sciences
University of Stirling**

November 1996

This thesis is dedicated to Jimmy M^cGregor

24/4/02 - 6/10/96

Contents

Abstract

Acknowledgements

List of Tables and Figures

Abbreviations

1	Introduction	1
1.1	Introduction	1
1.2	Protein folding- A history	2
1.3	The protein folding problem	4
1.3.1	<i>In vitro vs. in vivo</i>	5
1.4	Egg white proteins	7
1.5	Riboflavin binding protein	9
1.5.1	Structure of RfBP	11
1.5.2	Post-translational modifications of RfBP	12
1.5.3	Riboflavin binding and the binding site of RfBP	12
1.5.4	RfBP as a model system for studies of protein folding	13
1.6	Previous work	14
1.7	Aims of the project	14
2.	Experimental Section And Materials	15
2.1	Absorbance spectroscopy	15
2.2	Circular dichroism	15

2.2.1	Steady state circular dichroism	15
2.2.2	Stopped-flow circular dichroism	15
2.3	Fluorescence spectroscopy	16
2.3.1	Steady state fluorescence	16
2.3.2	Stopped-flow fluorescence	16
2.4	SDS PAGE	17
2.4.1	Molecular weight markers	17
2.5	Preparation of riboflavin stock solution	18
2.6	Estimation of RfBP and riboflavin concentration	18
2.7	Riboflavin fluorescence quenching assay	18
2.8	Preparation of guanidinium chloride solution	19
3	Purification and Characterisation of RfBP	20
3.1	Introduction	20
3.1.1	Spectroscopic analysis	21
3.2	Methods	23
3.2.1	Purification of holo-RfBP	23
3.2.2	Preparation of apo-RfBP	23
3.3	Characterisation of RfBP	24
3.4	Results	24
3.4.1	SDS PAGE	24
3.4.2	Binding of riboflavin	25
3.4.3	Spectroscopic analysis	25
3.5	Discussion	25

4	Unfolding and Refolding of RfBP	28
4.1	Introduction	28
4.1.1	The study of unfolding and refolding proteins	28
4.1.2	Monitoring the unfolding and refolding process	30
4.2	Methods	31
4.2.1	Unfolding of apo-RfBP	31
4.2.2	Refolding of apo-RfBP	32
4.2.3	ANS as a probe for structural changes in RfBP	33
4.3	Results	33
4.3.1	Unfolding of apo-RfBP	33
4.3.2	Refolding of denatured RfBP (steady state analysis)	35
4.3.3	Refolding of denatured RfBP (stopped-flow analysis)	37
4.3.4	ANS binding	38
4.4	Discussion	38
5	Reduction and Reoxidation of Disulphide Bonds in RfBP	42
5.1	Introduction	42
5.1.2	Disulphide bonds in RfBP	43
5.1.3	Protein disulphide isomerase	43
5.2	Methods	46
5.2.1	Reduction of RfBP	46
5.2.2	Estimation of free sulphydryl groups	47
5.2.3	Reoxidation of reduced RfBP	47
5.2.4	The effect of PDI on the reoxidation of RfBP	47
5.3	Results	48

5.3.1	Reduction and reoxidation of apo-RfBP	48
5.4	Discussion	50
6	Dephosphorylation of RfBP	54
6.1	Introduction	54
6.1.2	Phosphorylation of apo-RfBP	55
6.2	Methods	56
6.2.1	Dephosphorylation of RfBP	56
6.2.2	Inorganic phosphate determination	56
6.2.3	Phosphate stain for SDS gels	57
6.2.4	Characterisation and refolding of dephosphorylated RfBP	58
6.2.5	ANS as a probe for structural changes in dephosphorylated RfBP	58
6.3	Results	58
6.3.1	Dephosphorylation of RfBP	58
6.3.2	SDS gel analysis	59
6.3.3	Spectroscopic analysis	59
6.3.4	Refolding of dephosphorylated RfBP	59
6.3.5	ANS binding	60
6.4	Discussion	60
7	Deglycosylation of RfBP	62
7.1	Introduction	62
7.1.2	Glycosylation of apo-RfBP	63
7.1.3	Deglycosylation of RfBP	64

7.2	Methods	65
7.2.1	Chemical deglycosylation of apo-RfBP	65
7.2.2	SDS PAGE analysis of deglycosylated apo-RfBP	66
7.2.3	Characterisation of deglycosylated RfBP	68
7.3	Results	68
7.3.1	SDS PAGE analysis of deglycosylated RfBP	68
7.3.2	Spectroscopic analysis of deglycosylated RfBP	68
7.4	Discussion	69
8	General Discussion	72
8.1	Summary of Results	72
8.2	Unfolding	74
8.3	Refolding	75
8.3.1	CD - regain of secondary structure	75
8.3.2	Fluorescence - regain of tertiary structure	77
8.3.3	Slow phases in protein refolding	78
8.4	The effect of post-translational modifications on the refolding of RfBP, and relevance to <i>in vivo</i> folding	81
8.5	Future work	82

Appendix 1

References

Abstract

Hen egg riboflavin binding protein (RfBP) acts as a source of riboflavin to the developing embryo. It is the most abundant vitamin binding protein in the egg white. Mutations giving rise to a lack of RfBP lead to embryo death at approximately 13 days. RfBP binds riboflavin tightly in a 1:1 ratio. On formation of this complex, the fluorescence of riboflavin is completely quenched; this quenching is thought to be due to the stacking of aromatic groups within the hydrophobic binding pocket. This quenching provides a convenient assay for the integrity of the riboflavin-binding site of the protein.

RfBP consists of a single polypeptide chain of 219 amino acids of molecular mass 29.2 kDa. RfBP undergoes a number of post-translational modifications, namely: the formation of nine disulphide bonds, extensive glycosylation on Asn 36 and Asn 147, and the phosphorylation of eight serine side chains from between Ser 186 and Ser 197.

The unfolding and refolding of RfBP was studied by denaturing in 6 M guanidium chloride, followed by dilution in buffer, to start refolding. The processes were followed by both steady-state and stopped-flow circular dichroism and fluorescence spectroscopy. RfBP was found to readily unfold and refold, provided the disulphide bonds were intact. The regain of secondary structure was found to be too rapid to measure by the methods available (< 12 msec). The regain of tertiary structure was found to consist of 4 main phases, and a large proportion (80%) of the tertiary structure formed within 2 msec. The regain of riboflavin binding ability was complete at the end of the second phase, a reaction with a half-life of around 30 msec. In the presence and absence of riboflavin, the kinetics for the first 3 stages of tertiary structure

changes seemed to be identical. In the presence of riboflavin, however, seemed to impede the completion of the final, very slow stage, with the refolding reaction only going to 95% completion. The dephosphorylation of the protein seemed to have no affect on this process. When the 9 disulphide bonds are reduced however, RfBP is unable to spontaneously reoxidise to a native-like state in the presence of an oxidised/reduced glutathione redox system. However, the addition of protein disulphide isomerase to the system increases significantly the yield of successfully reoxidised RfBP to about 50%. Attempts to prepare deglycosylated RfBP by chemical methods were unsuccessful since the treatment led to fragmentation of the polypeptide chain.

Acknowledgements

I would like to thank Professor Nicholas C. Price for giving me the opportunity to undertake this project in the first place, and for the continual support, inspiration and advice, scientific and otherwise over the past three years. His seemingly photographic memory and the range and depths of his knowledge have proved invaluable many times. Nick was always enthusiastic, always interested in results and above all, always found the time to see me, even when his own workload was very heavy, which was very often! Conversations with other PhD students have revealed just how good, and rare, a supervisor he was, dare I say it, one of a kind! I would also like to thank the University of Stirling and the B.B.S.R.C. for the provision of my studentship.

My family have also been the source of much encouragement and support over my time at Stirling as a whole, and I doubt I would have been able to do it without them. As well as financial support, they have also had to put up with car-borrowing, food-cupboard pilfering, occasional decimation of the wine "cellar" and countless meals scrounged off them - I'm sure they realise that the development of a taste for the finer things in life is all part of a young man's education!

I feel as if I have been in the Department here forever, I feel so much a part of it is going to be really strange leaving. For the help, advice and friendship, many people deserve special mention. Sharon for being a sounding-board for some of my more off-the-wall theories, and for always knowing the right thing to say (as well as all the CD jiggery pokery!), Jacqui for the same, and for listening to all my moaning in the office, but most of all for little Robert - he must be ready for a "My First Scientist Kit" by now, I suppose it's just getting the lab coat

to fit him! Sue for putting up with my ranting. Loads of people for advice in the lab:- Doris (although her "rib-tickling" dialysis trick has got nothing on mine!), Kay, Hazel, Mary, Irina and countless others. Scott, Ronnie, Willie and Derek for help and advice on numerous matters, mostly non-science, above and beyond the call of duty. Apart from Nick, the other lecturing staff have always been on hand to help- Lewis Stevens (for all the good ideas and for letting me use 'his eggy protein' in the first place!), Mick North, whose courage and strength has been an example to us all, Grant Reid, and more recently, Tim Wess, Tim Whalley, Mike Wyman and Paul Bates, who have brought a breath of fresh air to the Department. Jacqui, Kay and Sue also deserve medals for sharing an office with me, I don't know where all my 'stuff' comes from, its just self-perpetuating junk! The room will seem empty without me.....

Robert Freedman and Stephen McLaughlin deserve thanks for the generous gift of the PDI, and for discussions relating to it.

One of my favourite aspects of the last three years has been the demonstrating. It has been a great pleasure to follow some of the students through their time here, and I hope the help and advice I offered was useful (and correct!). In particular, the class of 1996 deserve special mention: Jim (and Abi), Lorna, Kirsten, Elaine, Pauline, Debbie, Andrew, Joe, Nikki, the 2 Richards, Lynn, Colin (Hamilton boys!) Stephen, Steven, Fraser and Alistair. I have rarely met a finer bunch of people. It has be a great pleasure to meet them, and I hope we can keep in touch and stay friends.

Lastly, but not least, I have to acknowledge all the great friends that I have made during my time at Stirling, of whom I only have room to mention a few. A huge thank you must go to

Dale and Liz, and Kitt and Johnny, for all their support especially during the traumatic last 6 months, when I needed them most, not to mention giving me a bed to sleep in over the past 3 months- this thesis would have been even harder to write had I been living under canvas! Finally, in no particular order, Ben, Cheesy (Paul!), Sean, C.J., Neil, Scott, Lara, Gillian, Kirsten, Jane, Sara, Hedger, Andy, Chris, Craig, Eugene, Dave, all the other jakes and countless others, apologies to anyone I should have mentioned, but haven't.

List of Figures and Tables

- Figure 1.1 The primary structure of riboflavin binding protein
- Figure 3.1 The quenching of riboflavin fluorescence upon binding to RfBP
- Figure 3.2 The absorption spectrum of apo-RfBP
- Figure 3.3 The far UV CD spectra of apo- and holo-RfBP
- Figure 3.4 The fluorescence spectra of holo- and apo-RfBP (excitation at 290 nm)
- Figure 3.5 The proposed binding site for riboflavin (from Choi and McCormick, 1982)
- Figure 4.1 Changes in the fluorescence of RfBP after incubation in 0-6 M Gdn HCl (excitation at 290 nm)
- Figure 4.2 Changes in the CD spectrum of RfBP after incubation in 0-6 M Gdn HCl
- Figure 4.3 The effect of Gdn HCl on the binding of riboflavin by RfBP
- Figure 4.4 Changes in ellipticity at 225 nm and the fluorescence emission at 350 nm induced by Gdn HCl.
- Figure 4.5 Refolding of RfBP - fluorescence emission at 350 nm following dilution of Gdn HCl
- Figure 4.6 Refolding of RfBP - fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence of equimolar riboflavin
- Figure 4.7 Regain of riboflavin binding ability - fluorescence emission at 520 nm following dilution of Gdn HCl
- Figure 4.8 Fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence and absence of riboflavin

- Figure 4.8a Fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence and absence of riboflavin (kinetic fits)
- Figure 4.9 Fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence and absence of riboflavin
- Figure 4.9a Fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence and absence of riboflavin (kinetic fits)
- Figure 4.10 Riboflavin fluorescence quenching by native and refolding RfBP - changes in emission at 520 nm
- Figure 4.10a Riboflavin fluorescence quenching by native and refolding RfBP - changes in emission at 520 nm (kinetic fits)
- Figure 4.11 The fluorescence spectrum of ANS, in the presence and absence of apo-RfBP
- Figure 4.12 The fluorescence spectrum of apo-RfBP, in the presence and absence of ANS
- Figure 4.13 The fluorescence spectrum of holo-RfBP, in the presence and absence of ANS
- Figure 4.14 The quenching of riboflavin fluorescence by RfBP in the presence and absence of ANS
- Figure 4.15 Changes in fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence and absence of ANS
- Figure 4.16 Regain of riboflavin binding ability in the presence and absence of ANS- fluorescence changes at 520 nm following the dilution of Gdn HCl
- Figure 5.1 The far UV CD spectra of native and reduced RfBP

- Figure 5.2 The fluorescence spectra of native and reduced RfBP
- Figure 5.3 The quenching of riboflavin fluorescence by native and reduced RfBP
- Figure 5.4 The effect of PDI on the regain of riboflavin binding ability
- Figure 6.1 The roles of the 95kDa receptor protein and cathepsin D in the uptake and modification of RfBP, and other yolk proteins (from Stevens, 1996)
- Figure 6.2 Standard curve for free phosphate estimation
- Figure 6.3 Enzymatic release of phosphates from apo-RfBP using potato acid phosphatase
- Figure 6.4 The far UV spectra of native and dephosphorylated RfBP
- Figure 6.5 The fluorescence spectra of native and dephosphorylated RfBP
- Figure 6.6 The quenching of riboflavin fluorescence upon binding to dephosphorylated RfBP
- Figure 6.7 Changes in the CD spectrum of dephosphorylated RfBP after incubation in 0-6 M Gdn HCl
- Figure 6.8 Changes in the fluorescence spectrum of RfBP after incubation in 0-6 M Gdn HCl
- Figure 6.9 Fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence and absence of riboflavin
- Figure 6.10 The fluorescence spectra of ANS, in the presence of native and dephospho-RfBP
- Figure 7.1 The far UV CD spectra of native and deglycosylated RfBP
- Figure 7.2 Fluorescence spectra of native and deglycosylated RfBP
- Figure 7.3 The quenching of riboflavin fluorescence by native and

deglycosylated RfBP

Table 1.1	Main egg white proteins, and their possible roles
Table 2.1	Solutions used for SDS PAGE gels
Table 4.1	λ_{\max} , and relative fluorescence intensity at λ_{\max} , from fluorescence spectra of RfBP at various pH levels
Table 4.2	λ_{\max} , and relative fluorescence intensity at λ_{\max} , from fluorescence spectra of RfBP in 0-9 M urea
Table 4.3	λ_{\max} , and relative fluorescence intensity at λ_{\max} , from fluorescence spectra of RfBP in 0-6 M Gdn HCl
Table 4.4	The regain of tertiary structure in refolding RfBP
Table 6.1	Absorbance values for phosphate standards
Table 6.2	Inorganic phosphate determination on samples taken during dephosphorylation of RfBP

Abbreviations

ANS 8-anilino-1-naphthalene-sulphonate

BSA Bovine serum albumin

CD Circular dichroism

CM Carboxymethyl

DEAE Diethylaminoethyl

DTT Dithiothreitol

EDTA Ethylene diamine tetra acetate

ER Endoplasmic reticulum

Fab Antibody fragment

FAD Flavin adenine diphosphate

FMN Flavin mononucleotide

Gdn HCl Guanidinium chloride

GSH Reduced glutathione

GSSG Oxidised glutathione

HF Hydrogen fluoride

kDa	Kilodalton
Mab	Monoclonal antibody
M_r	Molecular weight
Nbs ₂	5,5'-dithiobis-(2-nitrobenzoic acid)
N-CAM	Neural cell adhesion molecule
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PDI	Protein disulphide isomerase
PEG	Polyethylene glycol
P4H	Prolyl-4-hydroxylase
PPIase	Peptidyl prolyl isomerase
RfBP	Riboflavin binding protein
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFMS	Trifluoromethanesulphonic acid
UV	Ultraviolet

Chapter1 Introduction

1.1 Introduction

The mechanisms involved in the production of proteins, a process fundamental to all living organisms, from the simplest single-celled microorganism to the blue whale, have been studied for many years. With the solving of the structure of DNA by Watson and Crick in 1953, the elucidation of the Central Dogma followed. From the transcription of the DNA gene sequence to the RNA template, the binding of anti-codon to codon, and the lengthening of the nascent polypeptide chain from the ribosome, these processes are well characterised and understood (Stryer, 1988). The final step in the pathway from DNA double helix to functioning protein, however, is not. The mechanism of folding a chain of amino acids into an ordered three dimensional structure, with biological function or activity, despite being studied for over 60 years, is still not fully understood. The resolution of the process of protein folding is therefore one of upmost importance, even if viewed from a purely academic point of view.

In the last 20 years the molecular biology revolution and the subsequent application of recombinant DNA technology has transformed our world (Watson *et al.*, 1987). With the potential to "cut and paste" genes from one organism to another, be it natural insecticides into plants, or human insulin into bacteria, it is vital that the theory behind folding, and also the mechanisms of the various enzymes implicated in folding *in vivo* are well characterised. Some problems have been encountered when prokaryotic expression machinery has to deal with the sequence for a eukaryotic protein, (especially if the protein is modified post-translationally, or is over-expressed, leading to

aggregation (inclusion bodies)) (Kane and Hartley, 1988; Marston, 1986). Comprehension of the various processes and factors involved are therefore necessary.

The solving of the protein folding problem will also benefit greatly other fields of protein research, such as structure determination. If the role of the amino acid sequence in dictating the final structure is known, then structure assignment shall become greatly simplified. In theory, knowledge of the primary structure should prove sufficient to predict the 3-D structure. Artificial polypeptides have been synthesised which adopt their predicted structure (i.e. β -barrel, helical structures) (Richardson and Richardson, 1989). These predictions are made on the basis of the tendencies some amino acids have to form either β -sheet or α -helix, or neither (i.e. proline is known to disrupt helices) (Chou and Fasman, 1978). Given the complexity of the average native protein, however, this prediction is not yet possible.

The field of *de novo* design would also be greatly advanced. The function and scope of artificial proteins that could be built from scratch is almost unlimited.

1.2 Protein folding- a history

The history of the unfolding and refolding of proteins is almost as long as and closely related to, the history of protein science itself. It was the result of the denaturing of proteins on exposure heat, that led to the first recognition of proteins as components of initially food, and later biological organisms and their products (Fruton, 1972). The substance to which egg white owed its heat-coagulable properties to was termed

"albumine" by Fourcroy in 1801, and a similar substance was also to be found in blood serum (Fruton, 1972). Attempts to find further examples led to a variety of methods being employed on biological samples, thus the acid-insoluble precipitate (*caseum*) found in milk after acidification was termed casein.

With the development of purification techniques, the coagulation process was studied in greater depth, and it was shown, by Chick and Martin, that the eventual precipitation on heating of albumin was preceded by an initial event they termed "denaturation". In 1931, Anson and Mirsky demonstrated that the denaturation of haemoglobin, to a state where it could not bind oxygen, differed spectrophotometrically from the native protein, and could not be crystallised, could be reversed under certain conditions, i.e. renatured (Fruton, 1972). It was also recognised around this time that the nature of these processes was probably due to the expanding and contracting of polymer chains, although not much was known about the composition and structure of proteins at this time.

The pioneer of the present day protein unfolding/refolding studies was Anfinsen, his studies resulting in a Nobel Prize for Chemistry in 1972. Shortly after Sanger had deduced the amino acid sequence of insulin, and thereby shown that the basic structure of a protein was a chain of amino acids of a unique sequence, Anfinsen showed that an unfolded protein could be refolded (Epstein *et al.*, 1963). This refolding led to the complete regain of biological activity, in the absence of any other factors, *in vitro*, thus demonstrating that the 3-D structure and conformation of a protein is determined solely by its amino acid sequence. He also speculated that "helper" proteins were present *in*

vivo, to aid the process. The role of chaperone proteins e.g. GroEL and BiP, and folding catalysts, such as protein disulphide isomerase (PDI) (discovered by Anfinsen's group in 1964) (Givol *et al.* 1964) and peptidyl proline *cis/trans* isomerase (PPI) (Fischer *et al.*, 1984) is recognised today, and although much of the practical work on folding involves "foldases", their mode of action, and the nature of interactions with their substrates, and also with other chaperones is far from completely understood.

1.3 The protein folding problem

Early proposals for the mechanism driving the folding event were largely based on thermodynamic considerations, namely that the protein molecule in its native folded state represented the global minimum energy state (Anfinsen, 1973). In 1969, however, Levinthal calculated that there are vastly too many possible conformations, even for a relatively short peptide chain, for folding to be a purely random search to attain this energy minimum, given that folding has to occur on a physiological time-scale (Zwanzig *et al.*, 1992). In fact, it has been estimated that for a polypeptide of 100 amino acids, it would take approximately 10^{66} years to sample all the possible conformations (10^{89}) (Creighton, 1994). Yet many proteins do fold readily, on a far smaller, biological timescale (msec-sec) (Tsong, 1976). To resolve this paradox, it was proposed that proteins fold via specific pathways, i.e the process is under kinetic control (Creighton, 1994a). Much of the work over the last 25 years has been directed towards identifying intermediates in such a pathway. Such intermediates are, by their very nature, transient, and their detection and characterisation has proved far from easy, but techniques such as pulsed hydrogen exchange, stopped flow CD etc, have been used to detect intermediates with specific secondary structures (Dobson *et al.*, 1992). However, the

refolding of simple, small proteins has been clearly shown to be under thermodynamic control- the unfolded and native state exist in thermodynamic equilibrium within the unfolding transition, with no evidence for intermediates. (Schmid, 1992).

Current theories ascribe to a combination of thermodynamic and kinetic control, a modification of the "jigsaw" theory of Harrison and Durbin (1985). They suggested that the folding process was analogous to completing a jigsaw puzzle- there is no set pattern or pathway, but folding can start anywhere, and follow innumerable pathways to reach the final point. The discovery of intermediates with specific secondary structure has led to the theory that early kinetic control (perhaps involving multiple pathways) of refolding produces a state in which the number of possible conformations which the refolding polypeptide would have to search through is significantly reduced, so the final structure can be attained in a physiologically reasonable time-scale (Šali *et al.*, 1994).

1.3.1 Folding *in vitro* vs. *in vivo*

Due to the myriad of macromolecules found in the cell, much of the research into protein folding is carried out *in vitro* (Freedman, 1992). Thus a homogenous population of protein molecules can be studied, under conditions which are easily altered (temperature, pH etc). One of the possible drawbacks of studying protein folding *in vitro*, is that the refolding involves a mature protein, the completed polypeptide chain, rather than the folding of a newly synthesised chain *in vivo* (Freedman, 1992). As well as the obvious differences between the two systems, the question of when folding actually begins *in vivo* has to be addressed, to gauge whether the study of refolding mature proteins is a valid model for the *in vivo* folding process, *i.e.* is folding co-

translational, or does it commence after the completion of the polypeptide chain?

Since most proteins are capable of refolding from a denatured state *in vivo*, it is fair to assume that folding does not depend on the initial presence of the N-terminal to prompt cooperative folding i.e. it is not controlled by the vectorial nature of translation (Freedman, 1992). There have also been examples of slightly truncated polypeptide chains being unable to refold- if 6 amino acids are removed from the C-terminal of BPTI, the protein is unable to refold and reoxidise, after reductive denaturation (Taniuchi, 1970; Andria and Taniuchi, 1978). Similarly, the removal of several C-terminal amino acids from staphylococcal nuclease severely perturbs the structure of the protein (Shortle and Meeker, 1989). A compact structure is formed, but it lacks native secondary and tertiary structure.

The question of additional cellular factors must also be addressed. In the test tube, refolding generally starts with a homogenous unfolded protein sample (in the random coil state, with a virtually limitless number of possible conformations), and a denaturing agent, such as Gdn HCl. The cell, on the other hand, would contain a myriad of molecules, native and folding proteins, chaperones, "foldases" etc, possibly acting in conjunction, as well as a host of other molecules unconnected with the protein folding processes. Although there is an apparent gulf between the two scenarios that has in the past caused doubt to be raised on the validity of studying the refolding of completed polypeptides *in vitro*, it has been shown that there are grounds for using such studies at least for small single domain proteins (Freedman, 1992). Furthermore, in the past decade, the use of chaperones and enzymes such as PDI and PPI *in vitro*, have

demonstrated the insights that *in vitro* refolding can give to the cellular process.

1.4 Egg white proteins

The egg white, or albumen, is formed by tubular glands found around the developing oocyte, during its passage through the oviduct, prior to the deposition of the shell by the shell gland (White and Merrill, 1988). In a typical hen egg, the white comprises roughly 60% of the volume, and contains approximately 50% of the total egg protein (Gilbert, 1971). This volume decreases as the embryo develops, over the three week incubation period, till at hatching none remains. Proteins are a major component of egg white, up to 10.5% by weight (Gilbert, 1971). A small amount of egg protein is used as an energy source for the developing embryo, but most of the protein present in the egg is converted into body protein by hatching. This would suggest that egg protein is mainly present as an amino acid reservoir for the embryonic chick. However, the conserved amino acid sequences of the egg white proteins characterised indicate otherwise (Stevens, 1991), as conserved sequences would indicate conserved structures, and hence function.

Although there are thought to be over forty proteins present in the albumen, only about a quarter of these have been well characterised. Egg white proteins have often been the focus of protein biochemists, especially in the early days of the discipline, mainly due their ready availability, the quantities present, and their ease of separation. Indeed many important discoveries and advances have been made through the study of egg white

proteins. The first atomic resolution three dimensional structure was derived from lysozyme, which led to the proposal of its catalytic mechanism (Phillips, 1967; Imoto *et al.*, 1972; Hammes, 1982). Introns, non-coding sequences within genes, were first found in ovalbumin (the most abundant egg white protein) (Breathnach, 1977), and the affinity of avidin for biotin is one of the strongest, specific ligand binding reactions known, a feature which has been exploited by biotechnologists, e.g in affinity chromatography and drug delivery (Stevens, 1991). Somewhat surprisingly though, the exact biological functions for most of the proteins in the albumen, other than a purely nutritional role, has yet to be ascertained. A summary of the postulated roles of some of the egg white proteins is given in Table 1.1 (adapted from Stevens, 1996).

As can be seen in Table 1, the majority of the egg white proteins appear to have an anti-bacterial/microbial role (Stevens, 1996). This is obviously very important to the developing embryo, in the absence of an comprehensive immunological system in the enclosed space of the egg (although a small amount of immunoglobulin, IgY, is present). As well as being a nutritional source for the chick, the proteins, vitamins and carbohydrate found in the white, are also just as useful a source of nutrition to bacteria and other microorganisms, but evolution has provided adequate defences against such attack. A number of ploys are utilised to this effect. The first barrier (after the shell) is the viscosity of the albumen, thickest at the shell due to ovomucin, which also protects against mechanical shock. A range of protease inhibitors exist, with different specificities, and in addition lysozyme degrades bacterial cell wall peptidoglycans. Finally the vitamin binding proteins are thought to act in this role as scavengers of free vitamins, denying them for microbial usage. The vitamin binding proteins are known

Table 1.1 : Main egg white proteins, and their possible roles

<u>Protein</u>	<u>Abundance (% of albumen)</u>	<u>Possible role</u>
Ovalbumin	54%	Metal ion binder/ protease inhibitor
Ovotransferrin	12%	Iron (III) binder
Ovomucoid	11%	Protease inhibitor
Lysozyme	3.4%	Anti-bacterial
Ovomucin	1.5%	Increases viscosity
Riboflavin binding protein	0.8%	Anti-microbial/ riboflavin transport
Immunoglobulin (IgY)	0.8%	Antibody
Ovostatin	0.5%	Protease inhibitor
Avidin	0.06%	Binds biotin/anti- microbial
Cystatin	0.05%	Protease inhibitor
Thiamine binding protein	trace	Binds thiamine

(adapted from Stevens, 1996)

to have another role, that of vitamin transport to the developing chick. Of these proteins the most abundant is riboflavin binding protein (Stevens, 1991).

1.5 Riboflavin binding protein

The biosynthetic pathways producing most vitamins were lost to the distant ancestors of vertebrates (White and Merrill, 1988). As a result mechanisms were developed to facilitate the uptake of the dietary vitamins, from the intestine and serum. To supply the developing embryo with riboflavin (vitamin B₂), vertebrates possess high affinity riboflavin binding proteins, which carry riboflavin to the appropriate area. While mammals can continuously supply the embryo with nutrients, avians, and other egg-laying species have to accumulate all the nutrients required for development prior to egg deposition. The riboflavin required for avian embryos is found in the yolk. The egg white of hens and their closer relatives is unique, in that it has a yellow tinge, due to riboflavin bound to RfBP. Other birds have egg white RfBP, but without bound riboflavin, and reptiles have neither RfBP or riboflavin in their egg white (White and Merrill, 1988). In the early 1950's, a commercial poultry breeder in Pennsylvania noticed that 3 otherwise healthy hens were laying fertilised eggs, which never hatched (White, 1990). Further study revealed that the embryos died at 13 days, and that these eggs lacked the characteristic yellow tinge found in normal egg whites. These hens were brought to the attention of Maw at Pennsylvania State University, who quickly concluded that the embryos were dying due to riboflavin deficiency, associated with a genetic flaw, and that they could only be rescued by directly injecting the eggs with riboflavin, which made it possible to perpetuate the mutant strain (White and Merrill, 1988; White, 1990).

In 1965, it was shown that the mutant hens lacked riboflavin binding protein, and that the uptake of dietary riboflavin was limited by the amount of RfBP present (Winter et al, 1967). More recently, it has been demonstrated that cDNA from the riboflavin deficient mutants has a 100 nucleotide sequence deletion in the messenger RNA. This was found to correspond to a complete exon from the RfBP gene missing, due to a G→A mutation at position 1 of the downstream 5' splice site, causing the loss of RfBP (MacLachlan *et al.*, 1993).

In 1966, Blum produced a model for the uptake of riboflavin in laying hens. He had isolated RfBP from serum, and concluded that it was virtually identical to the RfBPs already purified from egg white and yolk (see White and Merrill, 1988, and references therein). He proposed that the serum RfBP, synthesised in the liver, forms a complex with dietary riboflavin in the blood stream. This is then transported to the yolk. It is now thought that this transport occurs via a "piggyback" method, where RfBP binds to vitellogenin, which then crosses the vitellin membrane by a 95 kDa receptor protein (MacLachlan *et al.*, 1994). Upon entry to the yolk, a 13 residue peptide is cleaved from the C-terminal portion of RfBP by cathepsin D (Stevens, 1996).

Hen egg white riboflavin is synthesised in the oviduct. Although yolk RfBP is associated with riboflavin transport, it is thought that hen egg white riboflavin is primarily concerned with anti-microbial activity, through the scavenging of free riboflavin. Hen egg white contains holo-RfBP, but the RfBP present is not saturated. Indeed, it has been found that if apo-RfBP is injected into the eggs of *rd* recessive hens (RfBP negative mutants), the minute amount of riboflavin present is bound by the RfBP,

thus denying it to the embryo, which dies at an even earlier stage (2 days, as opposed to 13 for untreated *rdrd* embryos) (Lee and White, 1995).

1.5.1 Structure of RfBP

White, yolk and serum RfBP are all products of the same gene, with minor tissue specific differences in their post-translational modifications (White and Merrill, 1988). The protein is globular, monomeric and there is no evidence for separate domains. The primary structure has been deduced from direct amino acid sequencing (Hamazume *et al.*, 1984) and from cDNA sequencing (Zheng, 1988), and consists of 219 amino acids (fig 1.1). The N-terminal residue is pyroglutamic acid, and polymorphism can occur at residue 14, where 30-50% of molecules have lysine instead of asparagine (Hamazume *et al.*, 1984; Norioka, 1985).

RfBP is a phosphoglycoprotein (Rhodes *et al.*, 1959; Miller *et al.*, 1982). From the amino acid sequence, carbohydrate and phosphate composition, the total molecular mass is 29.2 Kda (Hamazume *et al.*, 1984). This is less than the value of 30-36kDa suggested from other methods (SDS PAGE, sedimentation equilibrium, etc) (see White and Merrill, 1988, and references therein). This discrepancy is probably due to the bulky carbohydrate groups present. Molecular weights of glycoproteins are also often over-estimated by SDS PAGE (Hamazume *et al.*, 1984). The isoelectric point of RfBP is about 4.0 (Rhodes *et al.*, 1959). There is, as yet, no 3-D structural information available for RfBP. Crystals that diffract to 2.8 Å have been obtained, but these proved unsatisfactory for structure determination (Zanette *et al.*, 1984).

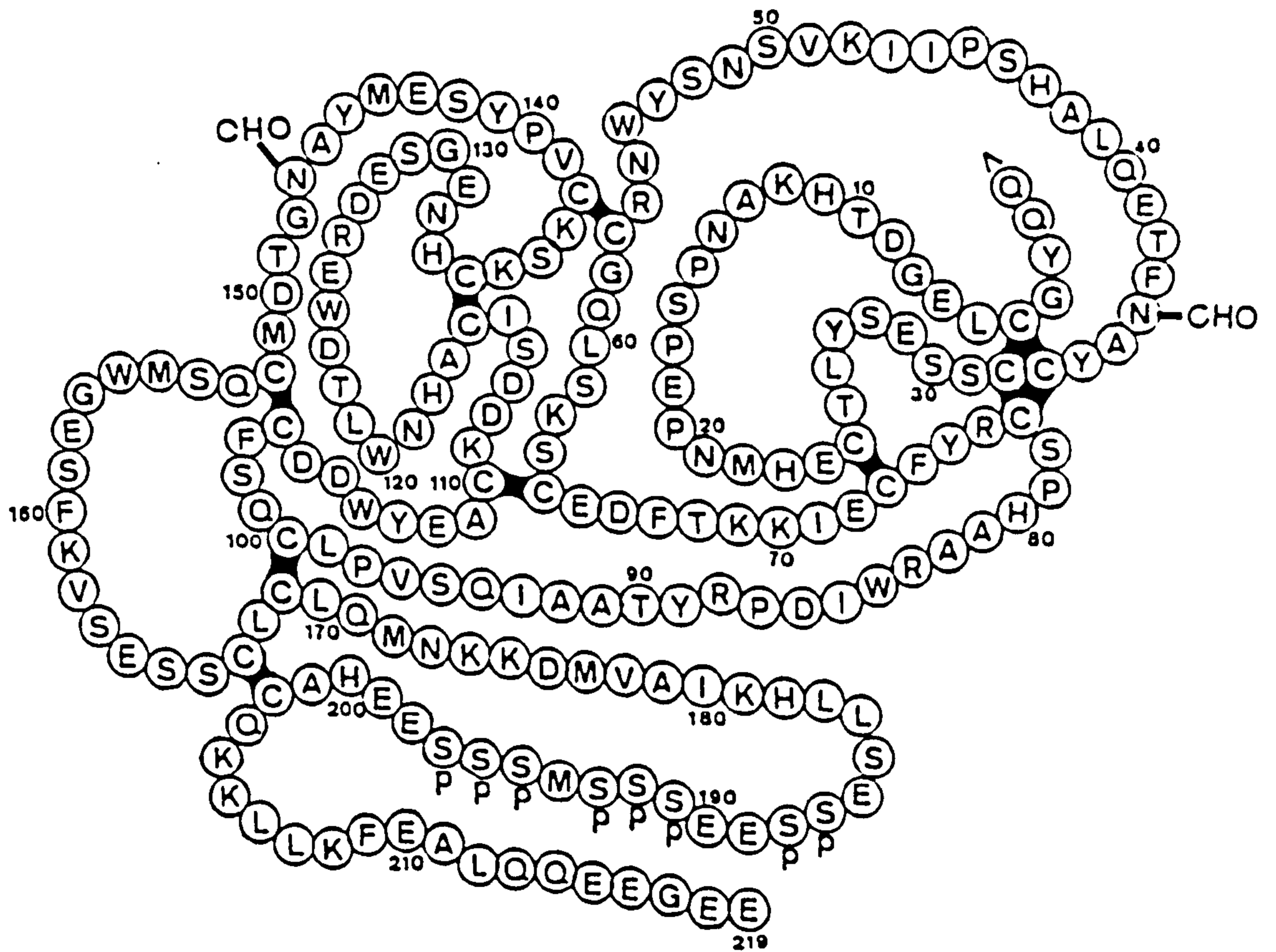


Figure 1.1 The primary structure of riboflavin binding protein (CHO- carbohydrate, P- phosphate) (from Hamazume *et al.*, 1987)

1.5.2 Post-translational modifications of RfBP

RfBP undergoes a number of post-translational modifications, namely phosphorylation, glycosylation, disulphide bond formation and limited proteolysis (Hamazume *et al.*, 1984). RfBP has 8 phosphorylated serines (Rhodes *et al.*, 1959; Miller *et al.*, 1982). These are thought to be involved with serum RfBP uptake to the yolk (Miller *et al.*, 1982). These are present in a highly anionic region between residues 186 and 199 which, in the egg white protein also includes five glutamate and one methionine residues (Hamazume *et al.*, 1984). The main differences between the egg white and yolk serum proteins are in the structure and composition of the carbohydrate (Miller *et al.*, 1982a). RfBP is extensively disulphide bonded, with 9 disulphide bridges (Kozik, 1982a). Limited proteolysis also occurs; the nucleotide sequence predicts an N-terminal 17 residue signal peptide and two arginine residues at the C-terminus, not observed in the mature protein (MacLachlan, 1993).

1.5.3 Riboflavin binding and the binding site of RfBP

Apo-RfBP binds riboflavin very tightly ($K_d = 1.3 \times 10^{-9}$ M), in a 1:1 ratio (Bevcar and Palmer, 1982). The avidin-biotin binding system (also found in egg white) is one of the few ligand binding systems with a lower K_d , itself only surpassed in strength by a few metal ligand binding proteins. Binding studies with flavin derivatives (FMN, FAD, etc) show that RfBP can bind other similar molecules, but not as strongly as riboflavin (Rhodes *et al.*, 1959; Bevcar and Palmer, 1982). These studies have shown that although no one part of the molecule is essential, the 7- and 8-methyl groups and the 2'-hydroxyl group contribute significantly to binding, while the hydroxyl groups on the N-10 side chain are thought to form hydrogen bonds with the protein in a tight cavity

(Choi and McCormick, 1980). The binding pocket is thought to form a hydrophobic cleft. One each of the six tryptophan and nine tyrosine residues have been shown to be essential for binding, through chemical modification studies (Blankenhorn, 1978). The tryptophan is not protected against chemical modification by bound riboflavin, and therefore could be near the hydrophobic binding pocket, while the tyrosine is protected, and probably lies within the binding site. Bound riboflavin prevents inactivation by carbodiimide, suggesting the presence of a carboxyl group at the binding site (Kozik, 1982).

An interesting feature of the binding system, is that upon the binding of riboflavin by the apo-protein, the fluorescence of riboflavin is completely quenched (Rhodes, 1959). This feature is thought to be due to a stacking interaction between riboflavin and an aromatic base at or near the binding site, most probably tryptophan (Choi and McCormick, 1980).

1.5.4 RfBP as a model system for studies of protein folding

There are a number of reasons why RfBP makes a good model in the study of *in vitro* protein folding. The protein is easily purified, in fairly large quantities. At 29.2 kDa, it is of a reasonable size. Other well studied refolding systems, have been smaller, *i.e.* BPTI, and lysozyme, are 6.5 and 14.3 kDa respectively, and contain fewer disulphide bonds (Jaenicke, 1987). The extensive post-translational modification undergone by RfBP (disulphide formation, glycosylation and phosphorylation) also offer scope for study- what role do these modifications play in the folding/refolding pathway? The quenching of riboflavin fluorescence upon binding to the apo-protein also provides a

convenient assay of biological activity.

1.6 Previous work

In preliminary work on the unfolding and refolding of RfBP, Allen *et al.* (1992) demonstrated that, provided the disulphide bonds remained intact, the protein could be refolded from its denatured form with high efficiency, when dialysis was used to remove the denaturing agent (Gdn HCl). In conditions under which the disulphide bonds in RfBP were broken and kept reduced, the unfolding of RfBP by Gdn HCl could not be reversed by subsequent dialysis.

1.7 Aims of the project

The main aims of the project were to :

- i)To purify riboflavin binding protein from hen egg white.
- ii)Characterise the refolding of denatured RfBP.
- iii)To assess the effect of the numerous post-translational modifications on the refolding process.
- iv)To assess the significance of the results in relation to models for protein folding.

Chapter 2 Methods

2.1 Absorbance spectroscopy

All single wavelength absorbance measurements were made on a LKB Biochrom Ultraspec II spectrophotometer, using a quartz 1 cm pathlength cuvette of capacity 1 ml, and the appropriate buffer as a reference blank. The absorption spectra were recorded on a Jasco V-550 UV/VIS dual beam spectrophotometer, using matched quartz 1 ml/1 cm pathlength cuvettes.

2.2 Circular dichroism

2.2.1 Steady-state circular dichroism

Steady-state far UV circular dichroism (CD) spectra (260-190 nm) were recorded on a Jasco J-600 spectropolarimeter, using cylindrical quartz cells of pathlengths 0.02 cm, 0.05 cm or 0.1 cm, depending on the concentration of the protein. Generally, spectra were recorded over the 260-190 nm range. Refolding studies measured the changes in the ellipticity at 225 nm against time.

2.2.2 Stopped-flow circular dichroism

Stopped-flow far UV CD studies were carried out on an Applied Photophysics SX-17MV Stopped-Flow Reaction Analyser instrument, using the umbilical cell in the 0.2 cm pathlength position. Unless otherwise stated, the mixing was in a 10:1 ratio (i.e. 11-fold dilution) of protein in 6 M Gdn HCl.

Refolding studies measured the change in ellipticity at 230 nm against time. 230 nm

was chosen as opposed to 225 nm, as reproducible results could only be obtained at 230 nm and above; at lower wavelengths the signal to noise ratio was considerably worse.

2.3 Fluorescence spectroscopy

2.3.1 Steady-state fluorescence

Fluorescence studies were recorded on a Perkin-Elmer LS-50B spectrofluorimeter in a 1ml cuvette of 1 cm pathlength, at 20°C. For protein fluorescence, the excitation wavelength was 290 nm, and the emission spectra were recorded over the range 300-400 nm. For riboflavin fluorescence studies, the excitation wavelength was 370 nm, with the emission recorded at 520 nm.

In the case of the refolding protein, the emission at 350 nm (with excitation at 290 nm) was measured against time (up to 2 hours). The quenching of riboflavin fluorescence by the refolding protein was measured by recording the emission at 520 nm (with excitation at 370 nm), against time. Slit widths were typically 5 nm + 5 nm bandpass, but were adjusted to cater for different protein/riboflavin concentrations.

2.3.2 Stopped-flow fluorescence

Fluorescence stopped-flow studies were recorded on the SX-17MV instrument, at 20°C. Protein refolding was recorded by emission at 350 nm (with excitation at 290 nm), against time. Quenching of riboflavin fluorescence was recorded at 520 nm (with excitation at 370 nm), against time. All slit widths were typically 1 nm, but were adjusted to cater for different protein/riboflavin concentrations. Unless otherwise stated, mixing was in a 10:1 ratio.

2.4 SDS-PAGE

Polyacrylamide gel electrophoresis was utilised, according to the modified discontinuous system of Laemmli (1970). The Bio-rad "Mini-Protean" II dual slab cell system was used.

The solutions, and volumes, used in the preparation of mini-gels can be seen in Table 2.1. Samples were diluted 1:1 with 2x sample buffer containing 2 μ l 2-mercaptoethanol/ml and boiled for 2 minutes. 10% of the sample volume of a 1:1 mixture of 1% bromophenol blue and 2-mercaptoethanol was added to the sample. Typically, 30 μ l of sample was loaded per lane with a Hamilton syringe. 150 V was applied across the apparatus until the bromophenol blue band was 0.5 cm from the bottom of the gel. The gel was placed in the stain solution for 30 min, then the destain solution for 2 hours, with several changes of solution.

2.4.1 Molecular mass markers

Dalton Mark VII molecular mass markers (purchased from Sigma) were used to estimate the molecular mass of proteins on the gel, by comparison of the relative distance of migration with the markers of known molecular masses. The marker proteins and their molecular masses are listed below:

<u>Marker Protein</u>	<u>Subunit</u>
	<u>M_r</u>
Bovine serum albumin	66000
Ovalbumin	45000
Glyceraldehyde-3-phosphate dehydrogenase (rabbit)	36000
Bovine carbonic anhydrase	29000
Soybean trypsin inhibitor	20000
α -Lactalbumin	14200

2.5 Preparation of riboflavin stock solution

Riboflavin (0.1 mg/ml) was partially dissolved in buffer (0.05 M sodium phosphate, pH6.8), and stirred vigorously for 30 min. The solution was then filtered twice, through Whatman No. 1 filter paper. The stock solution was stored in the dark at 4°C. Fresh stock solutions were prepared monthly.

2.6 Estimation of RfBP and riboflavin concentration

The concentrations of RfBP and riboflavin were determined by measuring the absorbances at 282 nm and 455 nm respectively and using the published extinction coefficients (Nishikimi and Kyogoku, 1973; Miller and White, 1986). The value for apo-RfBP at 282nm is $49000 \text{ M}^{-1}\text{cm}^{-1}$, and for riboflavin at 455nm, $12500 \text{ M}^{-1}\text{cm}^{-1}$.

2.7 Riboflavin fluorescence quenching assay

0.9ml of a riboflavin solution of known concentration was placed in a cuvette in the fluorimeter, and the fluorescence emission at 520 nm, with excitation at 370 nm, was

recorded. 10 μ l aliquots of an apo-RfBP solution, ten times more concentrated than the riboflavin (on a molecular basis), were added, the solution mixed well, and the fluorescence recorded as before, until the addition of RfBP had no further effect on the riboflavin fluorescence.

2.8 Preparation of guanidinium chloride solution

Gdn HCl solutions were made up by weight and the concentration was verified by measuring the refractive index of the solution (Nozaki, 1972). After correction for buffer, an 8 M solution of Gdn HCl has a refractive index increment of 0.132.

Chapter 3 Purification and Characterisation of RfBP

3.1 Introduction

Riboflavin binding protein (RfBP) was first isolated in 1958, by Rhodes and co-workers, who were fractionating egg white proteins using the new method of carboxymethyl-cellulose ion-exchange chromatography (Rhodes *et al.*, 1958). As well as providing higher yields of greater purity of the main known egg white proteins than traditional methods, they reported a previously unknown flavoprotein. The flavin moiety was found to be riboflavin. They proceeded to characterise the flavoprotein, which they termed riboflavin binding protein (Rhodes *et al.*, 1959)

The purification method used in this project is largely based on that by which Rhodes *et al.* (1958) first isolated the protein. A DEAE Sephadex column (anion exchange) is first used to isolate and purify the holo-protein. The apo-protein is purified by means of a CM Sephadex column (cation exchange). At pH 3.8, the holo-protein binds to the column, but the riboflavin dissociates from RfBP, leaving the apo-protein (Section 3.2.1).

RfBP has been isolated from a wide variety of sources (Walker *et al.*, 1991; Visweswariah *et al.*, 1987; Krishnamurthy *et al.*, 1984) from avian and reptilian eggs to pregnant mammals, including humans, although the hen egg white protein is probably the most widely studied, due to its availability and ease of purification.

3.1.1 Spectroscopic analysis

Several methods are available to study the conformation of proteins. Obviously, those which can produce a high resolution 3-dimensional picture of the protein are the most useful, but the techniques which are capable of this, NMR spectrometry and X-ray crystallography, require large amounts of protein (tens of milligrams) and a lengthy time-scale, possibly extending to years in the case of X-ray crystallography (Price, 1995). Both techniques have limitations; thus NMR is limited to smaller protein molecules up to about 25 kDa, while not all proteins will give crystals of a suitable size and quality necessary for diffraction studies. Spectroscopic methods, especially circular dichroism and fluorescence spectroscopy, on the other hand, provide reasonable, if less detailed structural information in a short time (less than 30 minutes), and require very little sample (100 μg of protein, or less) (Price, 1995). Due to the short time scale involved in data acquisition, further enhanced with the development of rapid mixing techniques (stopped-flow), it is even possible to record real-time structural changes within the molecule.

Circular dichroism (CD) involves the differential absorption of left and right circularly polarised components of plane polarised radiation, when a chromophore is chiral, or it has been placed in a chiral environment (Freifelder 1982; Price, 1995). Radiation is split into two circularly polarized components, by being passed through a modulator, such as a piezoelectric crystal, which is subject to an alternating electrical field. If the two components are not absorbed by the sample, or are absorbed by the same amount, then no CD signal will be produced. If, however, if one of the polarized components is absorbed to a greater extent than the other (e.g. due to the intrinsic or environmental

chirality of a chromophore), then the resulting radiation would be elliptically polarised, *i.e.* instead of a plane, an ellipse will be traced out. In practice the 2 components are not recombined after passage through the sample- the machine actually measures the change in absorbance. The term ellipticity (referring to the theoretical recombination) is widely used. From this degree of ellipticity, it is possible to relate the signal obtained to structural information, *i.e.* the relative proportions of α -helix and β -sheet can be determined from signals obtained in the far UV which arise from the peptide bonds.

Protein fluorescence is used as a measure of protein tertiary structure. Fluorescence occurs when a molecule is excited by radiation. The molecule then decays from its excited state, and the excess energy is released, at a higher wavelength. Protein fluorescence is largely due to the excitation of aromatic amino acid residues, particularly tryptophan, which has a maximum emission at 356 nm, after excitation at 290 nm (Harris, 1987). Burial within a protein, or shielding by other amino acid residues, will lead to a blue-shift in wavelength, thus revealing information on the location of these residues. The quenching of protein fluorescence, *i.e.* after ligand binding, can also reveal information on the active site, or the mechanism of action of the protein. During refolding studies, the regain of protein fluorescence can be used as an indicator as to the extent of regain of tertiary structure, given that the unfolded protein will have a maximum emission of 356 nm, which will shift to the blue on folding, as the tryptophan residues become buried or sheltered within the protein. With RfBP, the quenching of riboflavin fluorescence upon binding to the apo-protein (Rhodes *et al.*, 1959) is also useful, as an assay for the viability of the protein, *i.e.* after refolding or reoxidation (Allen *et al.*, 1992; Kozik, 1982a).

3.2. Methods

3.2.1 Purification of holo-RfBP

The purification schedule was based on that of Rhodes *et al.* (1958), with some modifications (Walker *et al.*, 1991). The whites of 12 eggs were separated by hand and pooled (approx. volume 400 ml). They were then homogenised, adjusted to pH 5 with glacial acetic acid, and spun in a Beckman GPR bench centrifuge for 15 min at 3000 g. The supernatant was then added to Sephadex A-50 (0.4 g per 100 ml of supernatant, pre-swollen in 0.05 M sodium acetate buffer, pH 5, for 3 hours), stirred once and left on ice for one hour. The gel was poured into a 10 x 3 cm glass column, and washed through with buffer (0.05 M sodium acetate, pH 5) until the A_{280} was less than 0.1. The column was then washed successively with buffer containing 0.05, 0.1 and 0.15 M NaCl, each time till the A_{280} was less than 0.1. Buffer containing 0.5 M NaCl was then applied to the column, and the eluted yellow RfBP collected in 10 ml fractions with a fraction collector. The absorbance of the fractions at 280 and 455 nm was measured, and those with an A_{455} above 0.02 were pooled and dialysed extensively against buffer. The A-50 column was washed with buffer containing 1 M NaCl (500 ml), then buffer (500 ml). The dialysed sample was loaded back on to DEAE A-50 column, and washed with the series of salt buffers as before. Following elution with 0.5 M NaCl buffer as before, the protein was dialysed against buffer.

3.2.2 Preparation of apo-RfBP

The pH of the sample was adjusted to 3.8. The sample was then loaded onto a 2 x 10 cm SP Sephadex C-50 column, previously equilibrated to pH 3.8. The column was

washed with 0.05 M sodium phosphate buffer, pH 3.8, until the yellow colour (riboflavin) was removed from the column, the bound protein forming an opaque white band, and the A_{455} was negligible (<0.005). The bound apo-protein was eluted with 0.1 M sodium acetate, pH 4, buffer containing 0.5 M NaCl, and collected in 5 ml fractions. These were dialysed against sodium phosphate buffer (0.05 M, pH 6.8) and the volume reduced approximately 8-fold by dialysis against 60% PEG. The protein was then freeze-dried, and resuspended to give a stock solution of the desired concentration (normally 10 mg/ml), in a final sodium phosphate buffer (pH 6.8) concentration of 0.05 M.

3.3 Characterisation of RfBP

The homogeneity and binding ability of the purified protein were assessed by SDS-PAGE (Section 2.4) (Laemmli, 1970) and a riboflavin fluorescence quenching assay (Section 2.7). Samples taken during the purification procedure were also analysed via SDS-PAGE. The absorbance, CD and fluorescence spectra of RfBP were recorded as described previously (Sections 2.1, 2.2.1, 2.3.1.) (the protein concentration was 0.1 mg/ml in each case).

3.4 Results

3.4.1 SDS PAGE

SDS PAGE analysis of samples (Section 2.4) taken at various points throughout the purification procedure showed the first DEAE-Sephadex column removed most of the unwanted protein, and the second column yielded pure holo-RfBP. The final SP Sephadex C-50 column was efficient in removing riboflavin from the holo-protein, to

RfBP, from 12 eggs.

3.4.2 Binding of riboflavin

The purified protein was found to bind riboflavin in a 1:1 ratio (Fig. 3.1), consistent with previous results. (Rhodes *et al.*, 1959; Allen *et al.*, 1992) The quenching was found to be linear with the concentration of RfBP, indicating tight binding (the K_d has been estimated to be 1.3×10^{-9} M (Bevcar and Palmer, 1982)).

3.4.3 Spectroscopic analysis

The absorption spectrum of 0.26 mg/ml RfBP (Fig. 3.2) showed that the absorbance peaked at 282 nm. The CD spectra (Fig. 3.3) of the holo- and apo-protein were almost identical, consistent with previous results (Allen *et al.*, 1992; Kumosinski *et al.*, 1982; Nishikimi and Kyogoku, 1973). The CONTIN secondary structure analysis program was applied to the CD spectra of apo-RfBP (Provencher and Glöckner, 1981). This analysis predicted the protein to contain 38% α -helix and 32% β -sheet. The protein fluorescence spectrum (Fig. 3.4) of the holo-protein, compared with that of apo-RfBP, showed a marked reduction on the intensity of fluorescence, by approximately 75%. The wavelength of maximum emission is also shifted slightly to the blue on formation of the holo-protein complex, from 345 nm to 343 nm (Fig. 3.4).

3.5 Discussion

The purification of RfBP from hen egg white was successful. Although 60mg was the

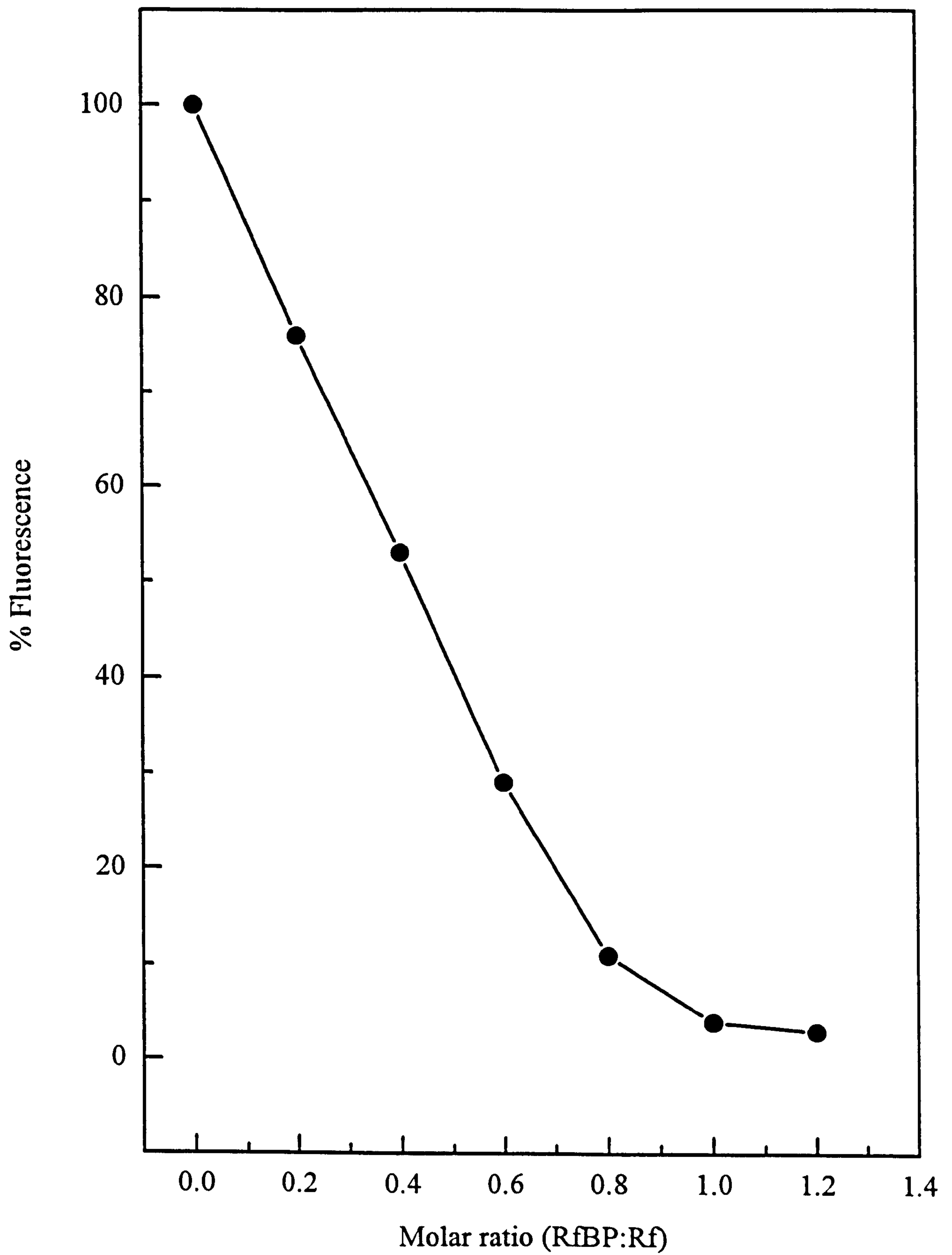


Fig 3.1: The quenching of riboflavin fluorescence upon binding to RfBP

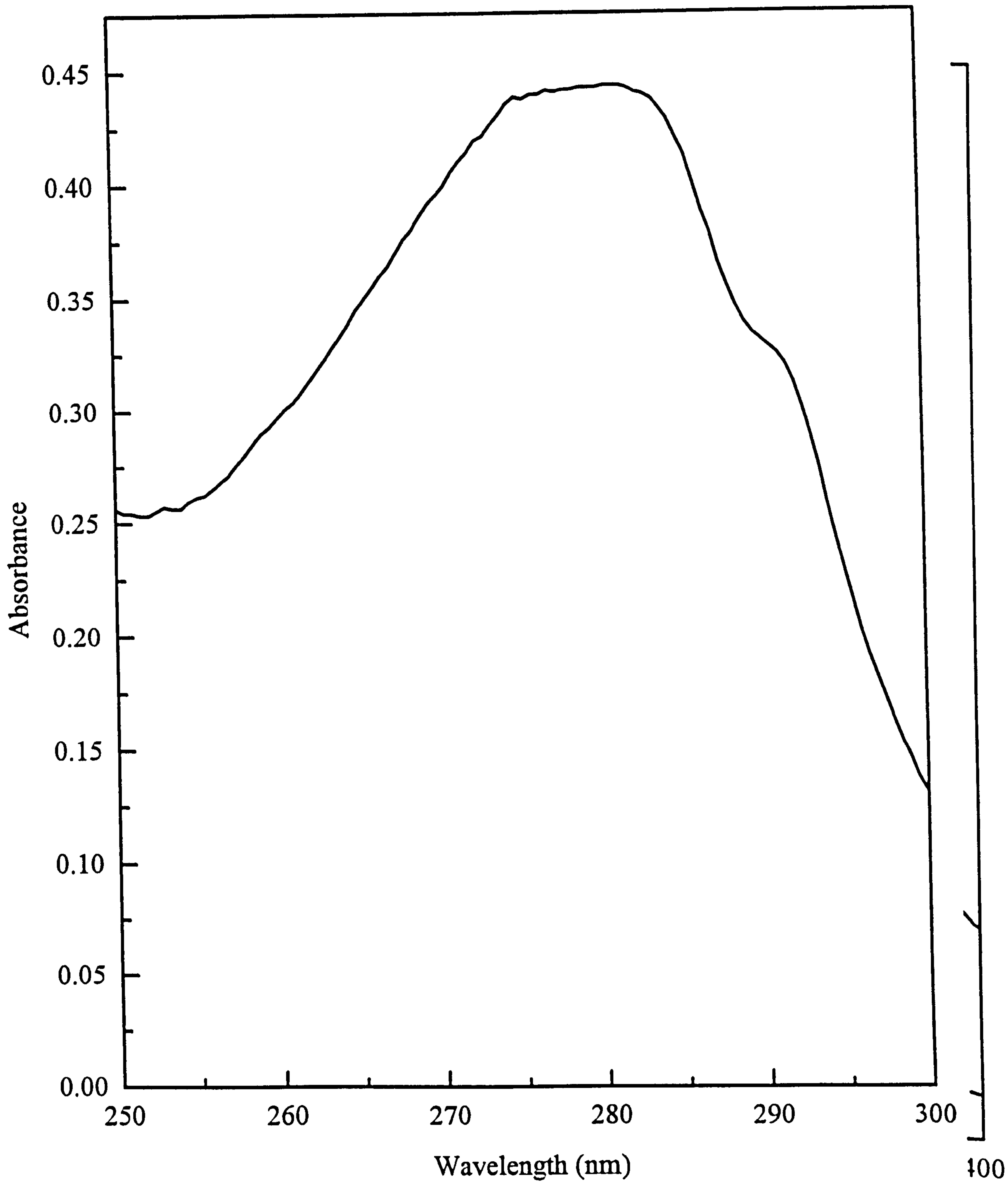


Figure 3.2 The absorption spectrum of apo-RfBP

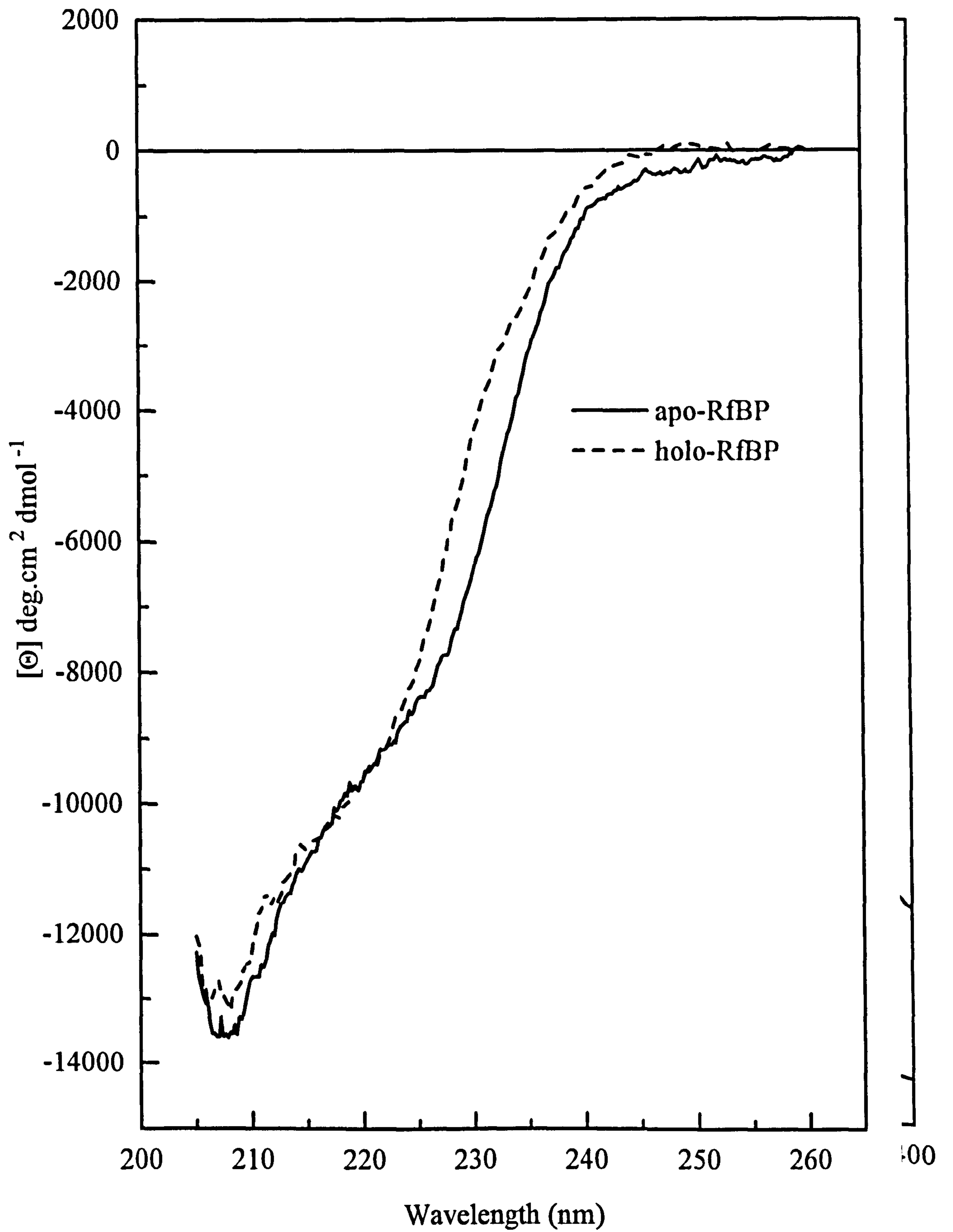


Figure 3.3 The far UV CD spectra of apo- and holo-RfBP

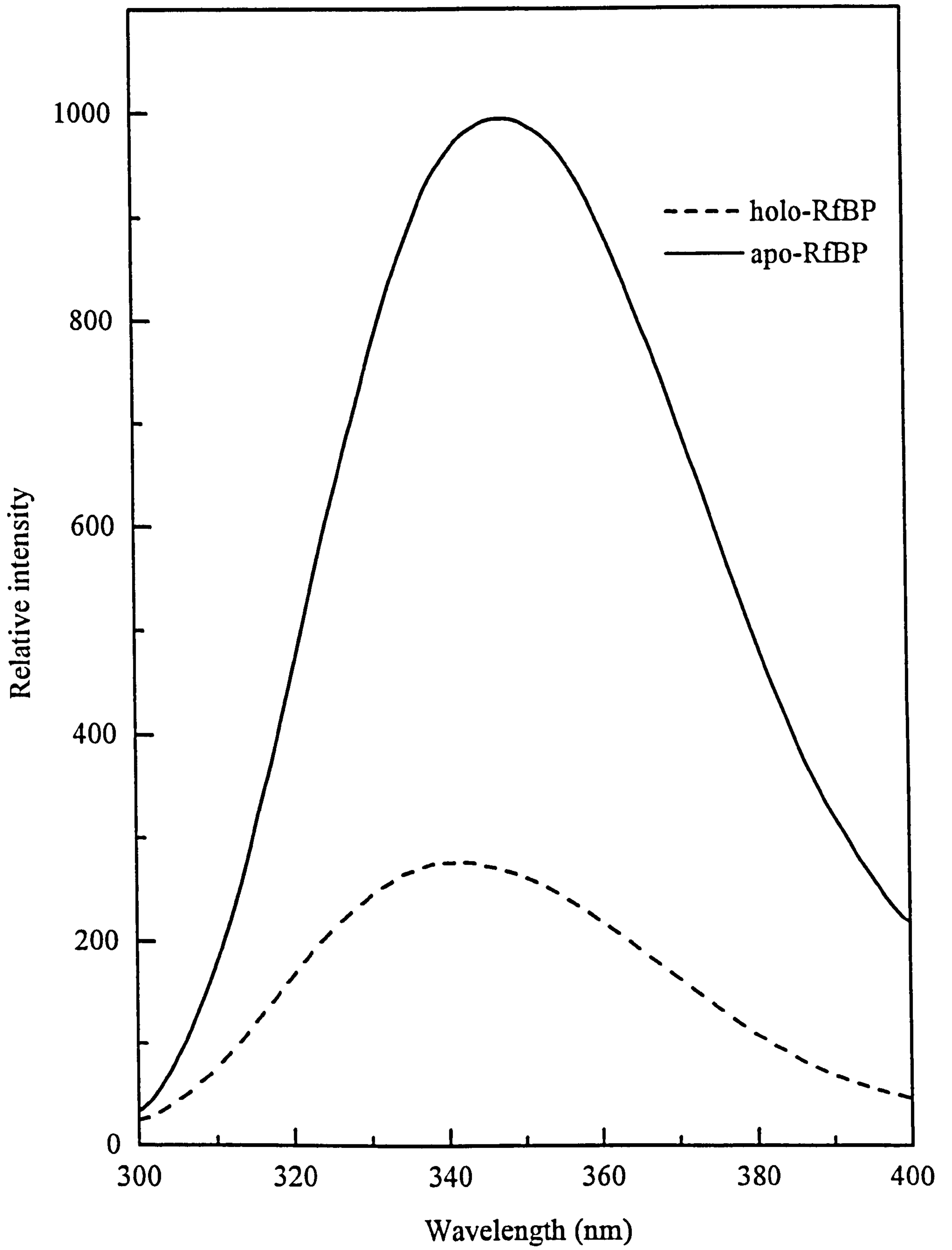


Figure 3.4 The fluorescence spectra of the holo- and apo-protein (excitation at 290nm)

The purification of RfBP from hen egg white was successful. Although 60mg was the average amount obtained from 12 eggs, on several occasions, nearly twice that amount was obtained. It was noted on these occasions that the egg white (after separation from the yolk) was much more intensely yellow. The reason for this is unclear, as it has been reported that, unlike some other egg white proteins, the abundance of RfBP is generally consistent.

RfBP was shown to bind riboflavin in a 1:1 ratio, above pH 4. Below this and the riboflavin dissociates from the protein, probably due to the disruption of hydrophobic interactions between the flavin molecule and the hydrophobic binding site. A wide range of riboflavin analogues have been shown to be able to bind to RfBP, indicating that no single part of the riboflavin molecule is essential for binding, although the pyrimidoid side of the flavin ring appears to play no part in the binding and is thought to be exposed to the solvent (Rhodes *et al.*, 1959; Choi and McCormick, 1980; Bevcar and Palmer, 1982)(Fig. 3.5). The two methyl groups on the dimethylbenzenoid portion are thought to be involved in hydrophobic interactions with the protein, while the hydroxyl groups on the N¹⁰ chain form hydrogen bonds with the protein, ensuring the tight binding of the flavin.

The complete quenching of riboflavin fluorescence upon binding to RfBP was noted. This is thought to be due to a ground-state stacking interaction between riboflavin and the tryptophan residue found in the binding site (through protection studies) (Blankenhorn, 1978). This would also account for the quenching of approximately 80% of the intrinsic protein fluorescence on riboflavin binding, and also the decrease in the

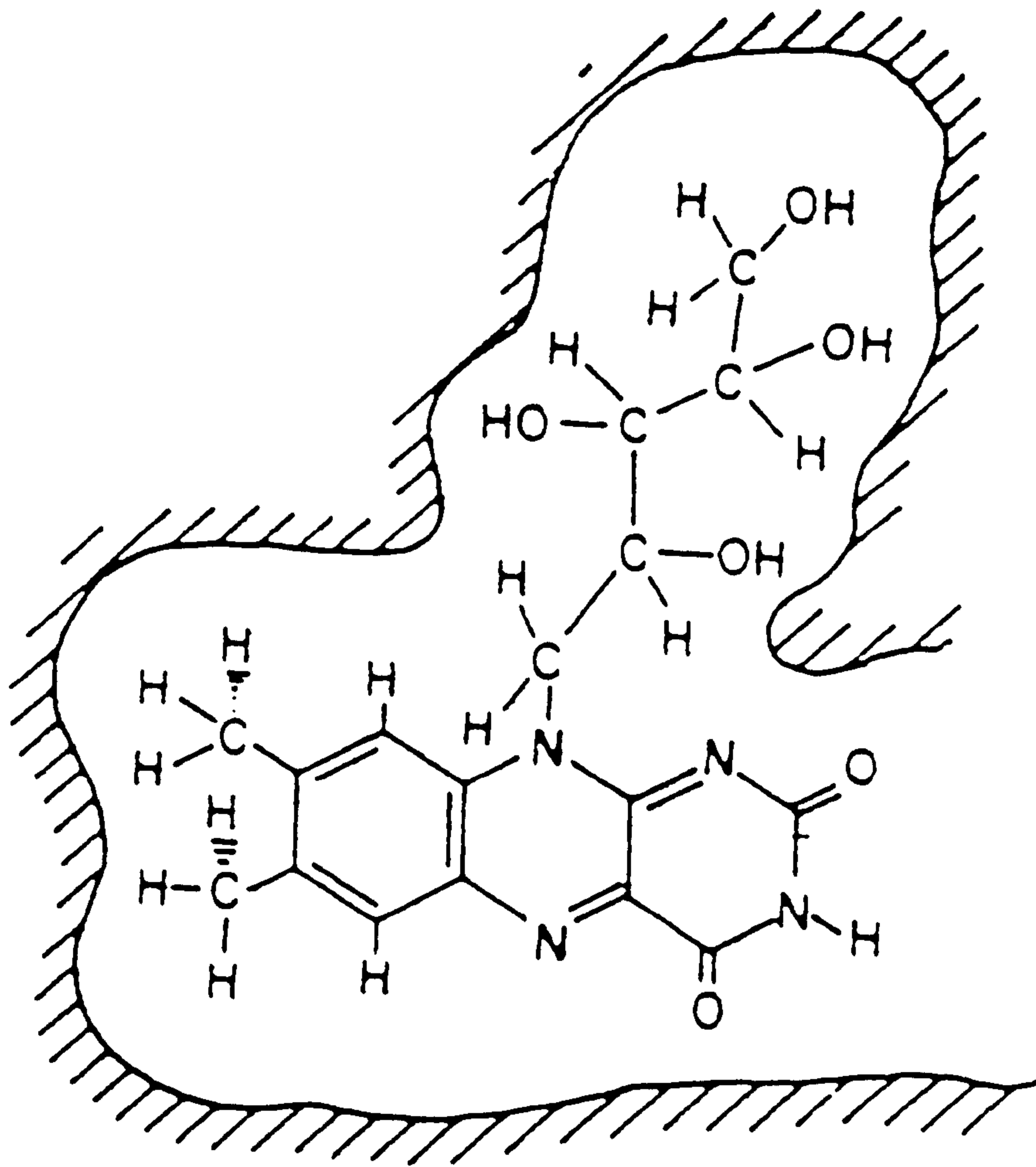


Figure 3.5 The proposed binding site for riboflavin (from Choi and McCormick, 1980)

maximum emission wavelength, as solvent accessibility of the tryptophan at or near the binding site was reduced.

Chapter 4 Unfolding and Refolding of RfBP

4.1 Introduction

The unfolding and refolding of oxidised RfBP was studied *in vitro* in order to characterise the folding process for this protein, and to provide a basis for understanding the folding process *in vivo*. Several methods of unfolding were tested, in order to find the most suitable for RfBP. The apo-protein was denatured and refolded in the presence and absence of riboflavin. The conformational state of the protein was ascertained via spectroscopy (CD and fluorescence) and binding ability. The kinetics of the folding reaction were determined using steady state and stopped-flow instrumentation. The use of ANS as a structural probe for the refolding process was also studied.

4.1.1 The study of unfolding and refolding proteins

The study of protein folding generally involves trying to understand the process by which a fully unfolded protein (U), matures into the folded native state (N), and identifying any intermediates that may lie between the 2 states (Creighton, 1993; Radford *et al.*, 1992; Bycroft *et al.* 1990). When unfolded, the protein is said to exhibit a random coil conformation (Tanford, 1968). The molecule resembles a randomly twisting chain with an essentially limitless number of conformations, although in practice, steric and spatial considerations, such as bond angles and degrees of rotation will reduce these (*i.e.* two residues cannot occupy the same space at the same time). None-the-less, it is plausible for every molecule in an average sample to have a different conformation at this stage (Creighton, 1993). This structure exhibits no stable secondary or tertiary structure, although there is some evidence for very short lived, fluctuating

elements of secondary structure (Murray-Brelief and Goldberg, 1989).

The fully folded structure is obviously the state of the native protein, exhibiting native secondary, tertiary (and quaternary if applicable) structure, and native biological activity, if relevant, *i.e.* catalysis, binding activity etc. What is of greatest importance to those workers interested in protein folding is, what lies between these two states, and by what means the random coil is converted to a folded compact molecule.

The molten globule has been postulated as an important intermediate in the protein folding "pathway" (Ohgushi and Wada, 1983). This is a state where the protein is reasonably compact, has native, or near-native secondary structure, but non-native, fluctuating tertiary structure. Molten globule formation is thought to occur early in the folding process of many proteins, as a result of a rapid hydrophobic collapse of the polypeptide chain (Mann and Matthews, 1993; Jones *et al.*, 1995).

8-anilino-1-naphthalene-sulfonate (ANS) is a commonly used probe for the molten globule state of proteins (Ptitsyn *et al.*, 1990; Semisotnov *et al.*, 1991). ANS binds to hydrophobic sites in proteins and the fluid tertiary structure of the molten globule provides many possible such sites. As the tertiary structure becomes more native-like, however, these sites/environments disappear, and ANS is released. As the fluorescence of ANS is markedly enhanced only upon molten globule binding, the increase and decay of ANS fluorescence has been used as probe for the transient molten globule state (Ptitsyn, 1992).

Several methods are available to unfold proteins *in vitro*, including changes of pH, heat and the use of chaotropic agents such as guanidinium chloride (Gdn HCl) and urea (Price and Stevens, 1989). Most proteins will rapidly denature in 6 M Gdn HCl, and so this is probably the most widely used method, although features of the certain proteins mean that the other methods can be used i.e a cold labile protein, unfolded by low temperature, could be refolded by return to an ambient temperature (Williams *et al.*, 1996; Nölting *et al.*, 1995). After denaturation in high concentrations of Gdn HCl, the polypeptide chain resembles a random coil, whereas proteins denatured by thermal or pH means have sometimes been shown still to have some secondary structure present. (Matthews, 1993), In the case of denaturing or chaotropic agents, the concentration of the agent can be reduced by dilution, to a level where little, or no, effect is seen on the protein. After denaturation, it is found that most small monomeric globular proteins will refold to a biologically active state spontaneously on return to suitable conditions.

4.1.2 Monitoring the Unfolding and Refolding Process

A number of methods are available to follow the unfolding and refolding of proteins. As previously discussed (Section 3.1.1), CD and protein fluorescence spectroscopy are powerful tools for analysing the conformations of proteins. The speed of data acquisition (especially with stopped-flow apparatus) means that the actual unfolding or refolding process can be followed by recording the change in CD/fluorescence signal at a certain wavelength as a function of time. Additionally, if the biological activity (eg binding of ligand, conversion of substrate to product) of the protein involves a spectroscopic change, the regain of this property can be followed. With RfBP, for example, the regain of riboflavin binding activity can be measured by recording the

fluorescence of riboflavin in the presence of the refolding protein. As the refolding protein regains activity, the riboflavin fluorescence is quenched.

4.2 Methods

4.2.1 Unfolding of apo-RfBP

4.2.1.1 Guanidinium chloride denaturation

An 8 M solution of guanidine hydrochloride was prepared and the concentration confirmed as described previously (Sections 2.8). Samples containing 0.1 mg/ml RfBP and Gdn HCl concentrations ranging from 0-6 M were prepared and incubated at 20°C for 15 minutes. The CD and fluorescence spectra were recorded for each sample as previously described (Sections 2.2.1, 2.3.1).

4.2.1.2 Urea denaturation

A 10 M solution of urea was prepared. Samples containing 0.1 mg/ml RfBP and urea concentrations ranging from 0-9 M were prepared and incubated for 15 minutes. The fluorescence spectrum was recorded for each sample as previously described (Section 2.3.1).

4.2.1.3 pH denaturation

A series of sodium phosphate buffers, from approximately pH 7-pH 1 were made up by titrating sodium phosphate with either NaOH or H₃PO₄. Apo-RfBP was added to a concentration of 0.1 mg/ml, and incubated for 15 minutes. The fluorescence spectrum was recorded for each sample as previously described (Section 2.3.1).

4.2.1.4 The effect of Gdn HCl on the structure and activity of RfBP

RfBP was incubated with Gdn HCl (0-6 M), for 15 mins at room temperature. Each sample was then subjected to CD and fluorescence analysis (Sections 2.2.1, 2.3.1) A riboflavin binding assay was then performed for each sample (Section 2.7)

4.2.2 Refolding of apo-RfBP

The protein was denatured, as described in 4.2.1.1, by mixing protein solution at an initial concentration of 10 mg/ml with 8 M Gdn HCl, to give a Gdn HCl concentration of 6 M, and incubated for 15 minutes. Refolding was initiated by dilution of the sample with buffer to reduce the Gdn HCl concentration. Dilution was carried out either manually or by stopped-flow mixing.

CD, protein fluorescence and riboflavin fluorescence were all recorded at the appropriate wavelengths as a function of time, as described in Chapter 2.

Experiments concerning the regain of secondary and tertiary structure during refolding were duplicated in the presence of an equimolar concentration of riboflavin.

With manual mixing (steady state), the final Gdn HCl concentration was 0.1 M, and for stopped-flow mixing, 0.54 M. The limit of 0.54 M was imposed on the stopped flow by the 1:10 mixing syringe setup employed. These concentrations of denaturant have a very minor effect on the protein, and do not affect binding of riboflavin.

To determine the start and end points of the refolding reactions, the

Table 4.1: λ_{\max} , and relative intensity of fluorescence spectra of RfBP at λ_{\max} , at various pH levels.

pH	λ_{\max} (nm)	Intensity (arbitrary units)
7.06	346.6	366
6.08	346.4	342
4.91	346.4	348
4.06	346.6	358
3.12	347.9	408
2.15	346.4	358
1.12	345.7	266

Table 4.2: λ_{\max} and relative fluorescence intensity at λ_{\max} , from
fluorescence spectra of RfBP in 0-9 M urea

[Urea] (M)	λ_{\max} (nm)	Intensity (arbitrary units)
0	346.2	396
1	346.4	396
3	347.9	445
5	348.9	505
7	352.3	795
9	354.2	977

Table 4.3: λ_{\max} , and relative fluorescence intensity at λ_{\max} , from
fluorescence spectra in 0-6 M Gdn HCl

[Gdn HCl] (M)	λ_{\max} (nm)	Intensity (arbitrary units)
0	345	189
1	345	209
2	347	263
3	350	313
4	352	322
5	353	330
6	356	384

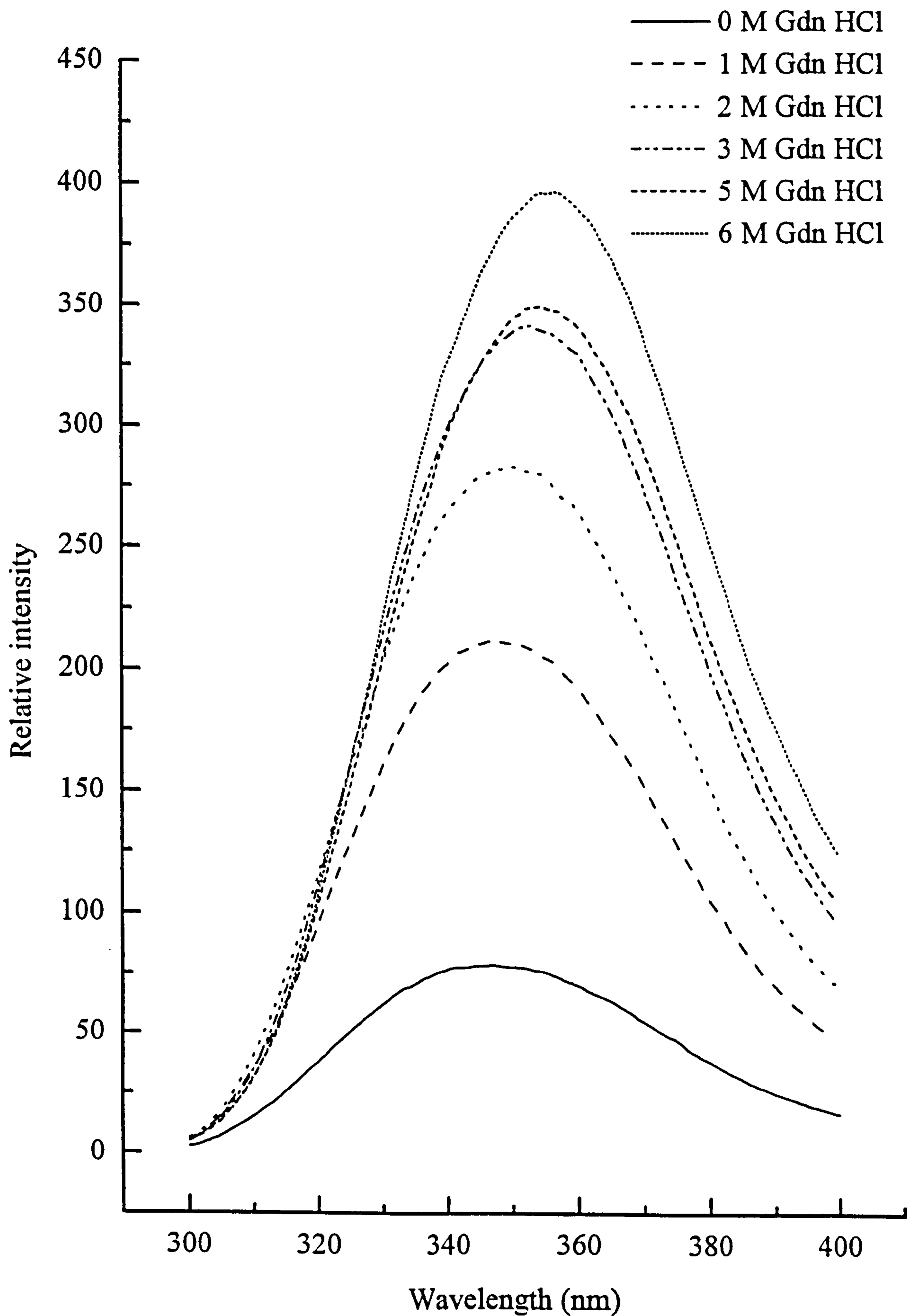


Figure 4.1 Changes in the fluorescence of RfBP after incubation with 0-6 M Gdn HCl (excitation at 290nm)

ellipticity/fluorescence intensity of RfBP were measured after incubation in 6 M Gdn HCl (start point) and either 0.1 M or 0.54 M Gdn HCl (endpoint), depending on the instrument used (steady state or stopped-flow).

4.2.3. ANS as a probe for structural changes in RfBP

Denatured RfBP was refolded as before, in the presence of 20 μ M ANS, and the fluorescence of ANS measured (excitation 370 nm, emission 480 nm), to test for the presence of a molten globule intermediate, through the enhancement of ANS fluorescence on binding to such a species. The reaction was studied with both steady state and stopped-flow instrumentation. The interactions between ANS and native RfBP was also investigated i.e. whether RfBP can bind ANS, and how the refolding process and riboflavin binding are affected by ANS.

4.3 Results

4.3.1 Unfolding of apo-RfBP

4.3.1.1 Fluorescence studies on various methods of denaturation

Tables 4.1, 4.2 and 4.3 show the wavelengths of maximum emission (λ_{\max}), and the relative intensities at each maximum. Figure 4.1 shows the fluorescence spectra recorded from denaturations involving increasing concentrations of Gdn HCl.

From a comparison of the three methods of unfolding tested, only Gdn HCl appears to be capable of unfolding RfBP completely. The value of λ_{\max} is the main indicator here- tryptophan has a λ of 356 nm- therefore if the fluorescence spectrum of the protein has

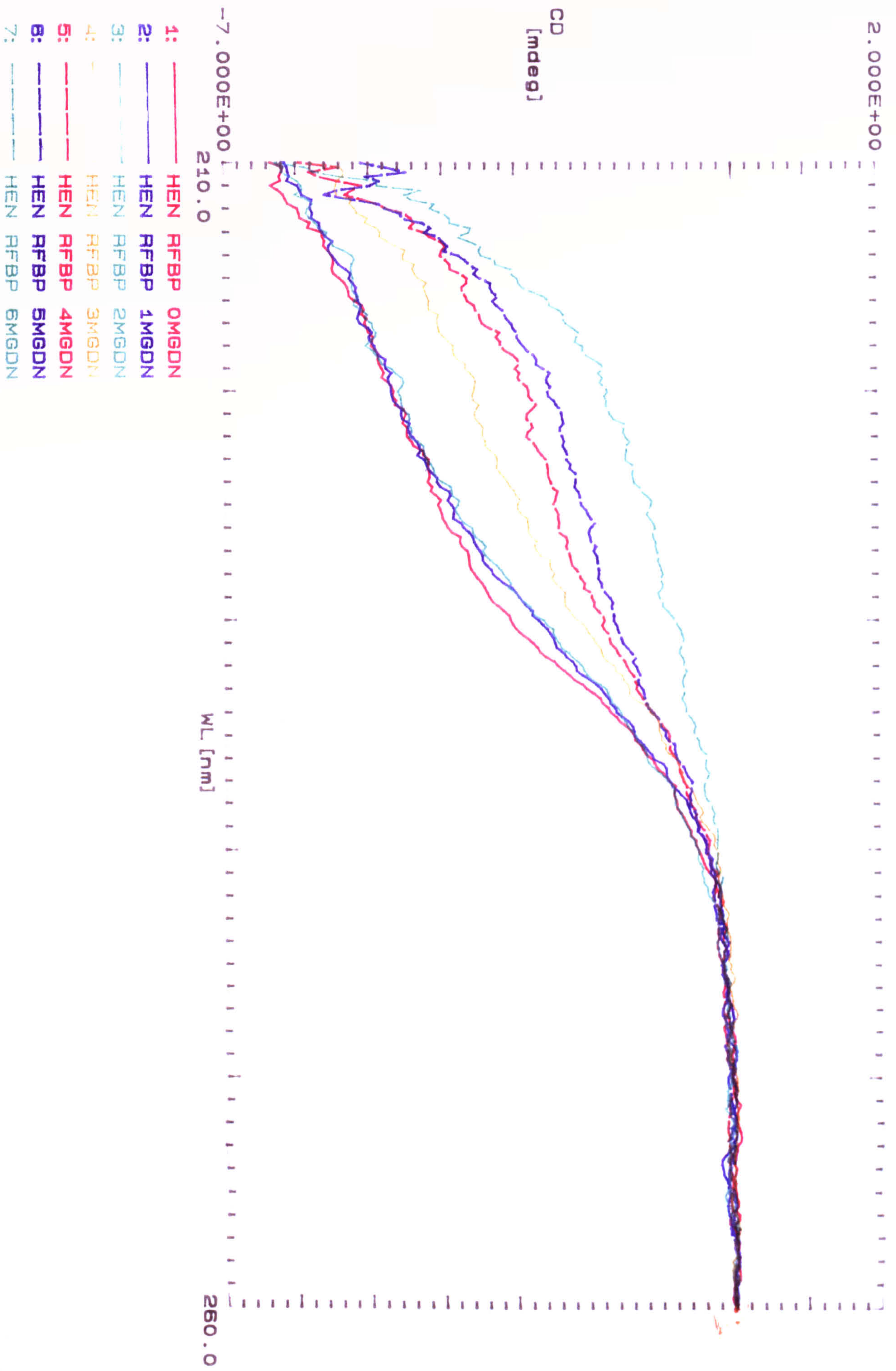
a λ_{\max} of 356 nm, then the tryptophans are considered fully exposed to the solvent (Harris, 1987).

The low pH has very little effect on the tertiary structure of RfBP, as can be seen in Table 4.1. The λ_{\max} is not affected by the pH, even as low as pH 1. In contrast, urea, especially at the higher concentrations, is more effective in RfBP denaturation, but at 9 M urea, the protein still has some residual tertiary structure, as the λ_{\max} is 354.5 nm, not 356 nm as would be expected if the protein was fully unfolded (Table 4.2). This can be seen in the presence of 6 M Gdn HCl ($\lambda_{\max} = 356$ nm), indicating that Gdn HCl is the best denaturant for RfBP of those tested (Table 4.3, Fig. 4.1).

4.3.1.2 Steady state CD and fluorescence analysis of RfBP after Gdn HCl denaturation

In agreement with the results of Allen *et al.* (1992), apo-RfBP appeared to be fully denatured after incubation in 6 M Gdn HCl; there was a total loss of secondary and tertiary structure and the riboflavin binding activity was lost (Figs. 4.2, 4.1 and 4.3). In 6 M Gdn HCl, the wavelength of maximum fluorescence emission is identical to that of tryptophan model compounds (356 nm), indicating complete exposure of tryptophan residues to the aqueous environment. Similarly, the CD spectrum above 205 nm (a lower limit imposed by the absorbance of Gdn HCl) of the protein in 6 M Gdn HCl is indicative of a random coil. This unfolding occurred despite the retention of the nine disulphide bonds in the protein. In agreement with the earlier data (Allen *et al.* 1992), the fluorescence and CD signal changes indicated that the unfolding was not a simple two-state process; a first change occurs between 0 M and 2 M Gdn HCl and a second

Figure 4.2 Changes in the CD spectrum of RfBP after incubation in 0-6 M Gdn HCl



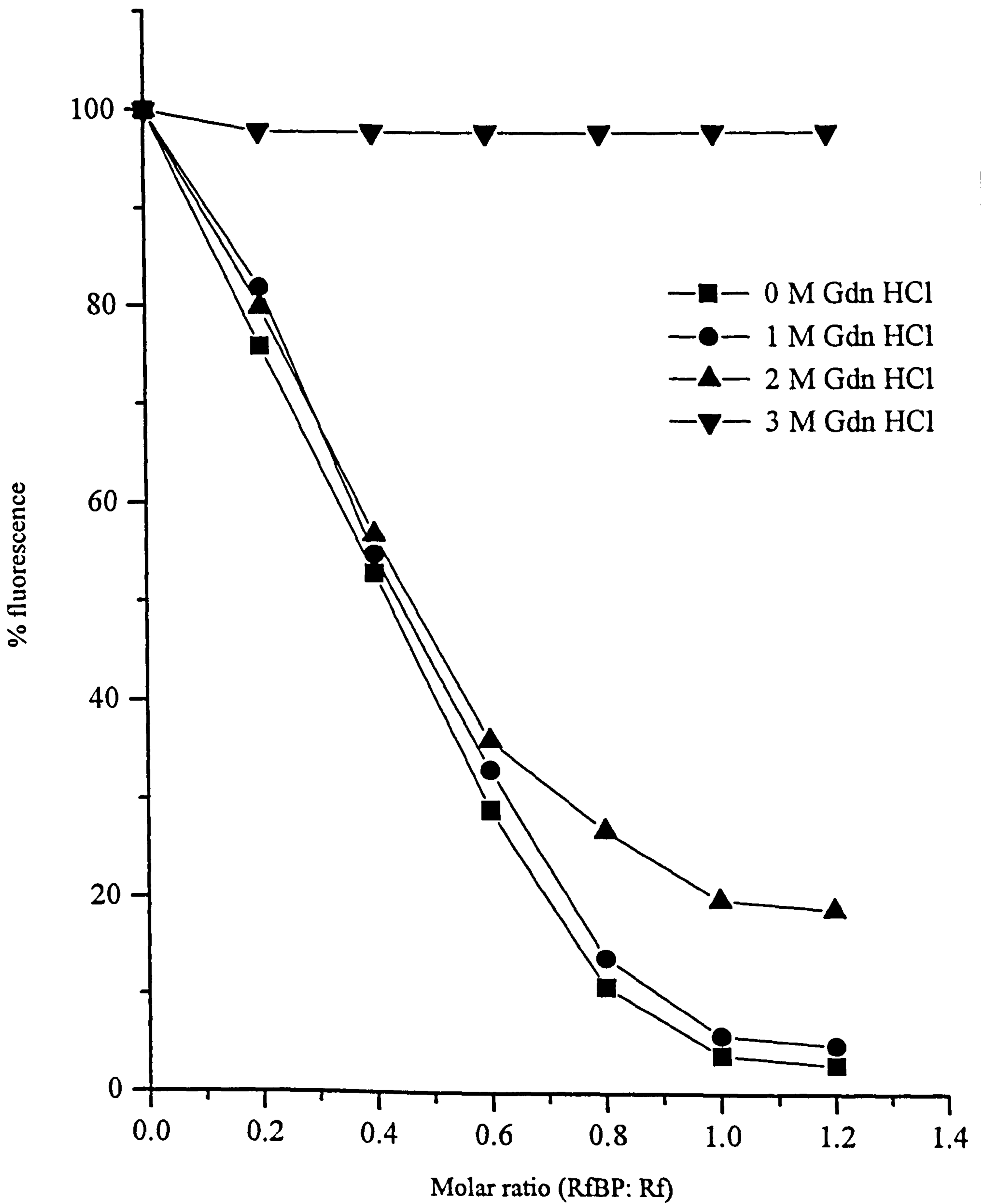


Fig 4.3: The effect of Gdn HCl on the binding of riboflavin by RfBP nc

between 5 M and 6 M Gdn HCl (Fig. 4.4). There appears to be an intermediate state of RfBP stable between 2 M and 4 M Gdn HCl.

4.3.2 Refolding of denatured RfBP (steady state analysis)

4.3.2.1 Changes in CD

The rate of regain of secondary structure of RfBP during refolding was examined by far UV CD. It was found that the changes in the ellipticity of the protein at 225 nm were complete within 10-15 s, the "dead time" of this type of manual mixing experiment. It was also shown that the presence of an equimolar concentration of riboflavin had no significant effect on the rate of changes in the far UV CD on refolding. These results indicated that the formation of native secondary structure during refolding was rapid.

4.3.2.2 Changes in protein fluorescence

The changes in protein fluorescence appeared to be at least biphasic. In the absence of riboflavin, 95% of the refolding (i.e. of the total fluorescence change) occurred within 50 seconds. This was followed by a slower phase, lasting up to 2 hours, in which the final 5% of the fluorescence change occurred (Fig. 4.5).

In the presence of equimolar riboflavin, only 90% of the tertiary structure was regained after the initial rapid phase, and a further 5% of the total change expected (i.e. the difference between RfBP in 6 M Gdn HCl and RfBP in 0.1 M Gdn HCl) occurred over the subsequent 2 hours (Fig. 4.6).

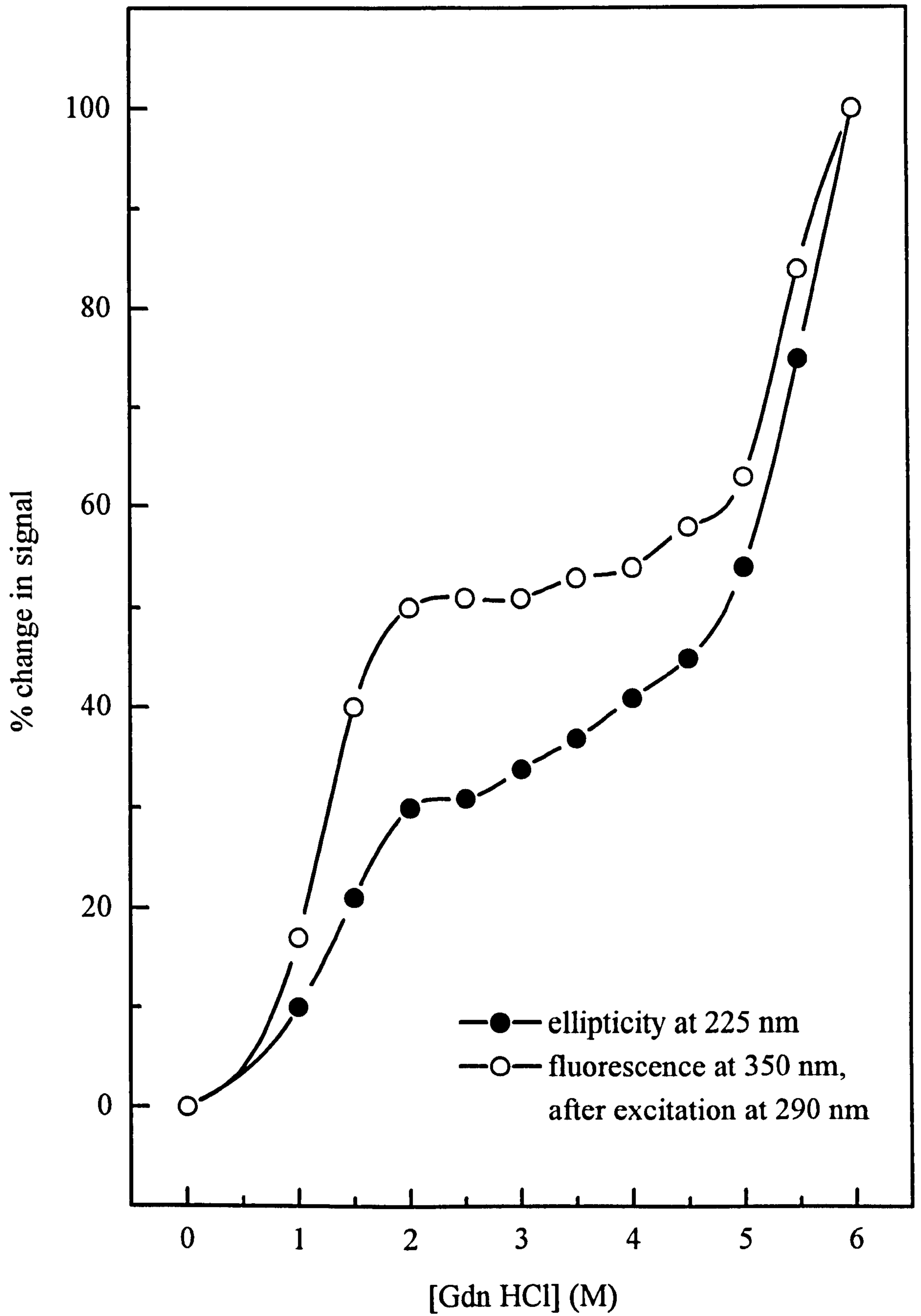


Figure 4.4 Changes in ellipticity at 225nm and fluorescence emission at 350nm induced by Gdn HCl

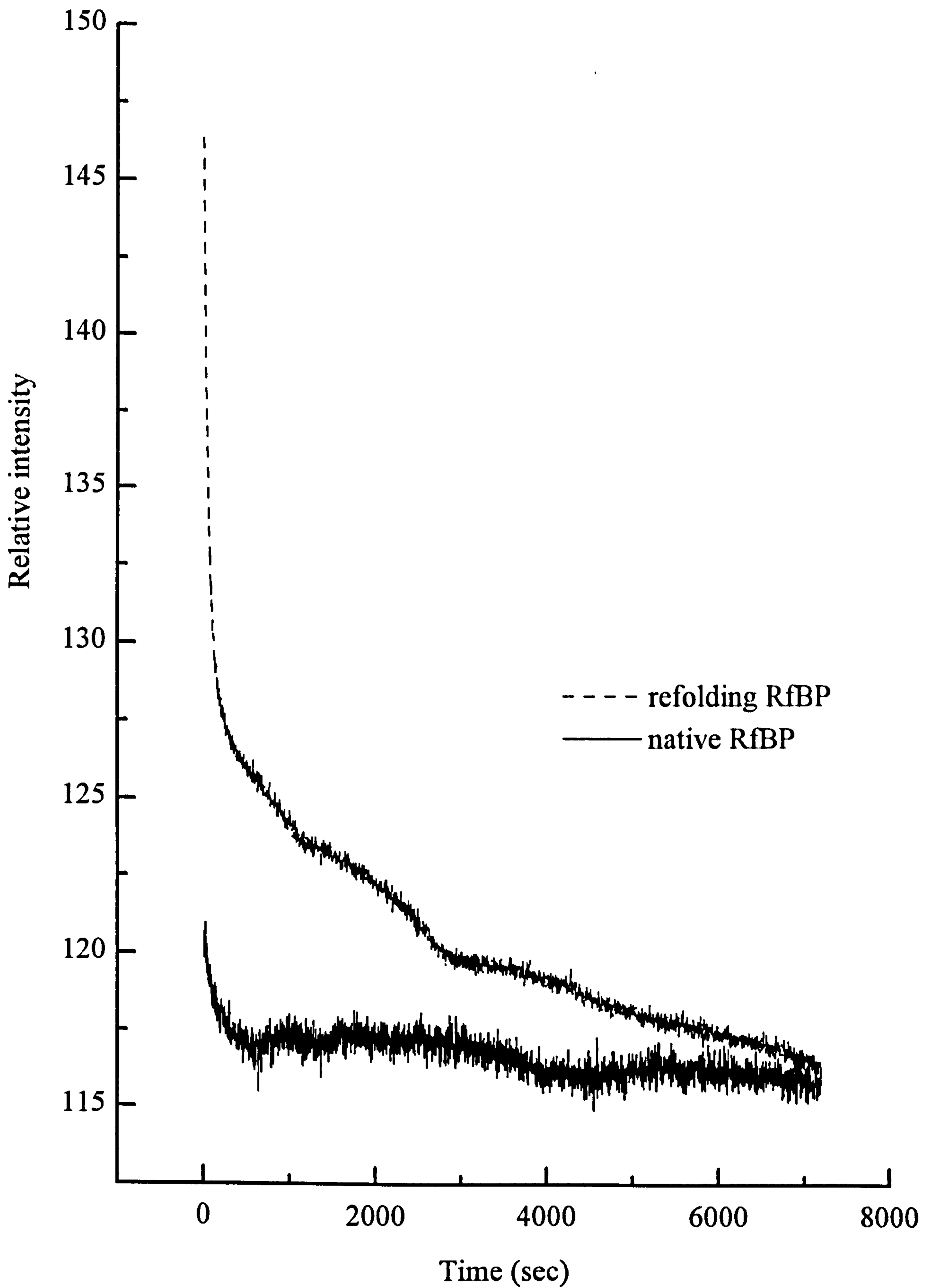


Figure 4.5 Refolding of RfBP - fluorescence emission at 350nm following dilution of Gdn HCl

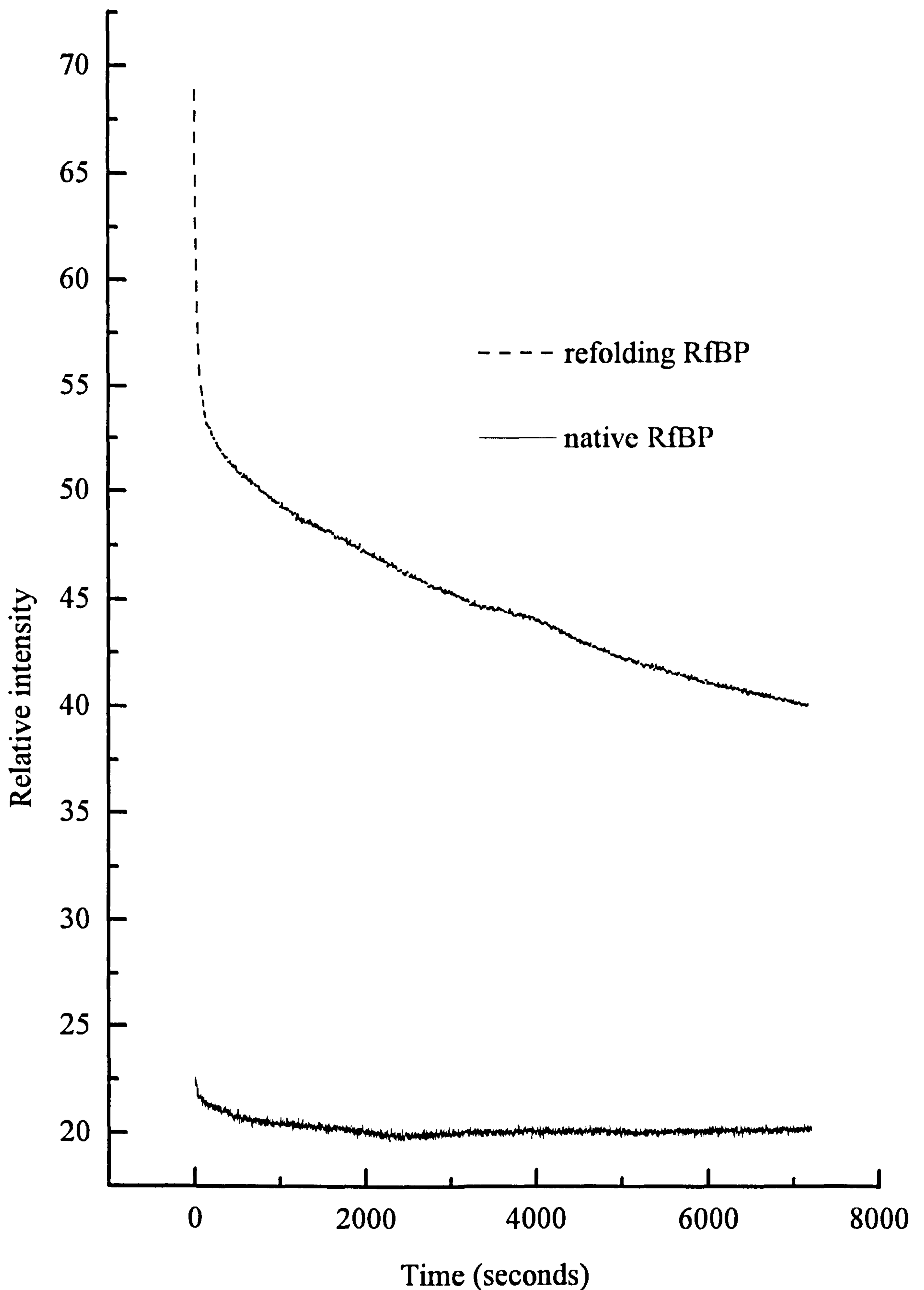


Figure 4.6 Refolding of RfBP - fluorescence emission at 350nm following dilution of Gdn HCl, in the presence of equimolar riboflavin

The slower phase, seen in both the presence and absence of riboflavin, could be indicative of some of the slower steps thought to be involved in protein folding, such as *cis/trans* isomerisation of X-Pro peptide bonds (Lang *et al.*, 1987). The results also seem to imply that the presence of riboflavin in some way slightly impedes the acquisition of the final native tertiary structure; this point is discussed later (Section 4.4).

4.3.2.3 Time course of riboflavin fluorescence quenching

In the riboflavin fluorescence quenching experiment, the native RfBP quenches the riboflavin fluorescence almost immediately, while the refolding RfBP takes almost 200 seconds to reach an endpoint, although the main part (> 90%) of the process is complete within 10 seconds (the 'dead time' of the experiment) (Fig. 4.7).

4.3.2.4 Summary of steady state analysis

From a combination of steady state fluorescence and CD studies, therefore, it would appear that the refolding denatured RfBP regains all of its secondary structure, and most of its tertiary structure, within a few seconds of the start of the refolding process, largely within the dead-time of the steady state instruments, approximately 10 seconds.

The completion of the regain of native tertiary structure took somewhat longer, up to one hour for the final 5%, in the absence of riboflavin. For a more detailed insight into the early stages of the folding process in which most of the structural changes occur, stopped-flow CD and fluorescence techniques were employed.

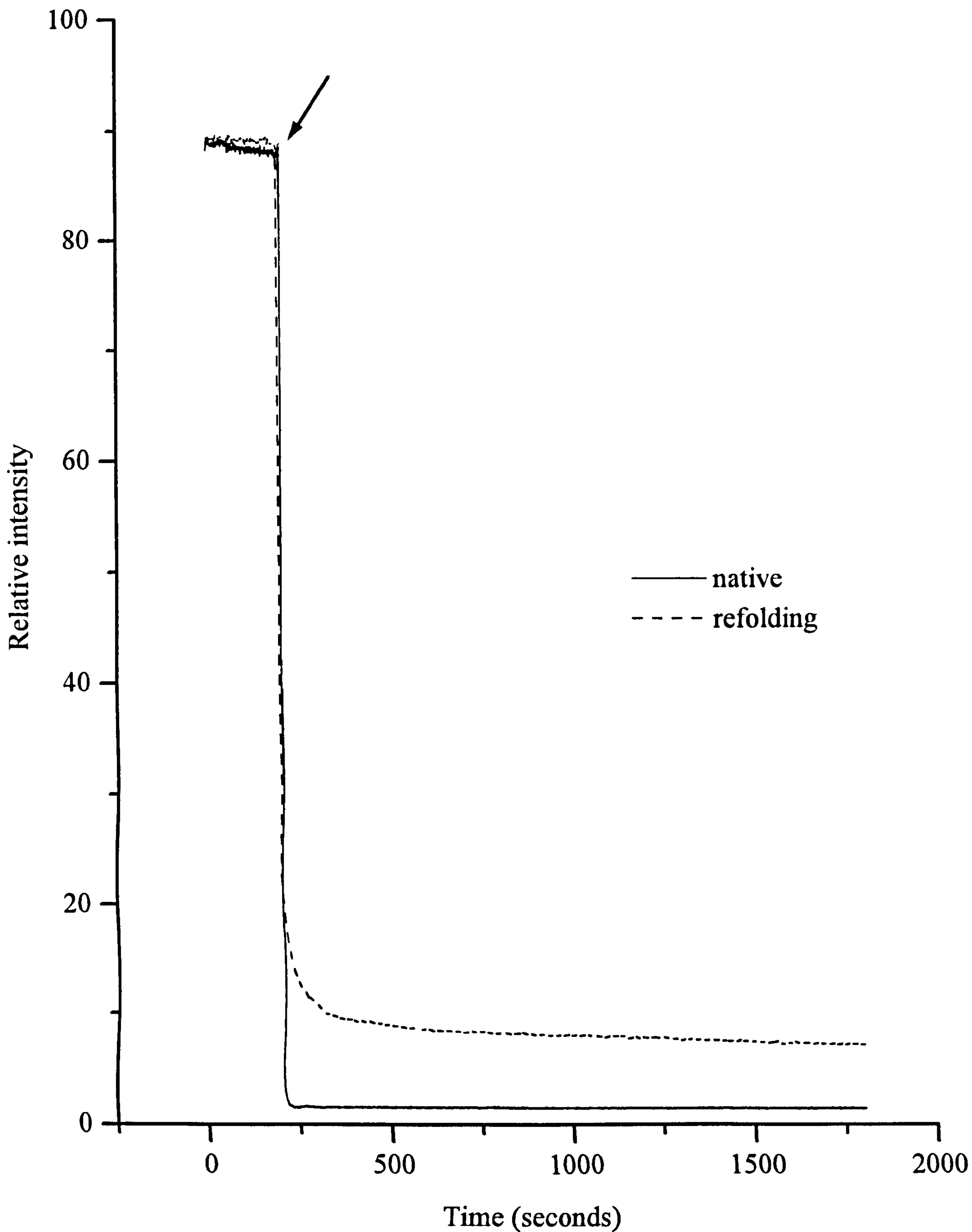


Figure 4.7 Regain of riboflavin binding ability - fluorescence emission at 520 nm following dilution of Gdn HCl (arrow marks dilution)

4.3.2.5 Refolding of denatured RfBP (stopped-flow analysis)

4.3.2.6 Changes in stopped-flow CD

The regain of secondary structure, as measured by the regain of CD signal at 230 nm was found to be complete within the dead-time of the stopped-flow instrument, which has been estimated to be approximately 12 msec (see Appendix 1). It was also shown that the presence of an equimolar concentration of riboflavin had no significant effect on the extent and rate of the changes in the far UV CD on refolding.

4.3.2.7 Changes in protein fluorescence

A total of 4 phases of fluorescence during the refolding were resolved, namely:- very fast, fast, slow and very slow (Figs. 4.5, 4.8, 4.9). Most (80%) of the fluorescence change again occurred in the dead-time of the instrument, estimated to be 1.7 msec (Appendix 1). The rates and half-times of the first three phases were calculated by the stopped-flow instrument's internal kinetic analysis software, while the half time of the the very slow phase was estimated from the steady state fluorimeter results (Table 4.4).

In the presence of riboflavin the kinetics of the 4 phases of protein fluorescence regain were identical to that of the refolding apo-protein, when the extra amplitude (approximately 80%) due to the quenching of the protein fluorescence by riboflavin was taken into account (Figs. 4.8, 4.9).

4.3.2.8 The regain of riboflavin binding activity

The regain of riboflavin binding activity was measured through the fluorescence change

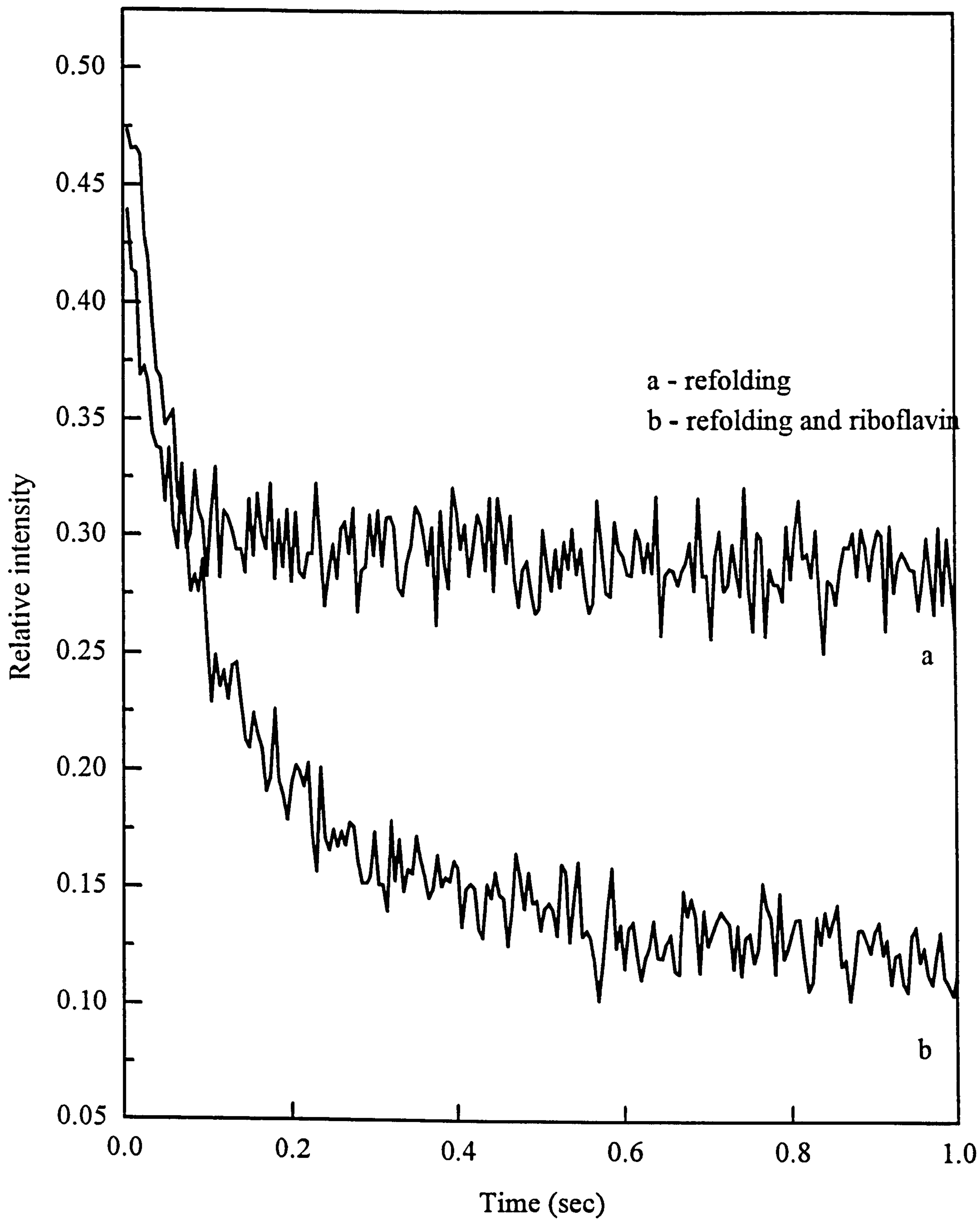


Figure 4.8 Fluorescence emission at 350 nm following dilution of GdnHCl, in the presence and absence of riboflavin

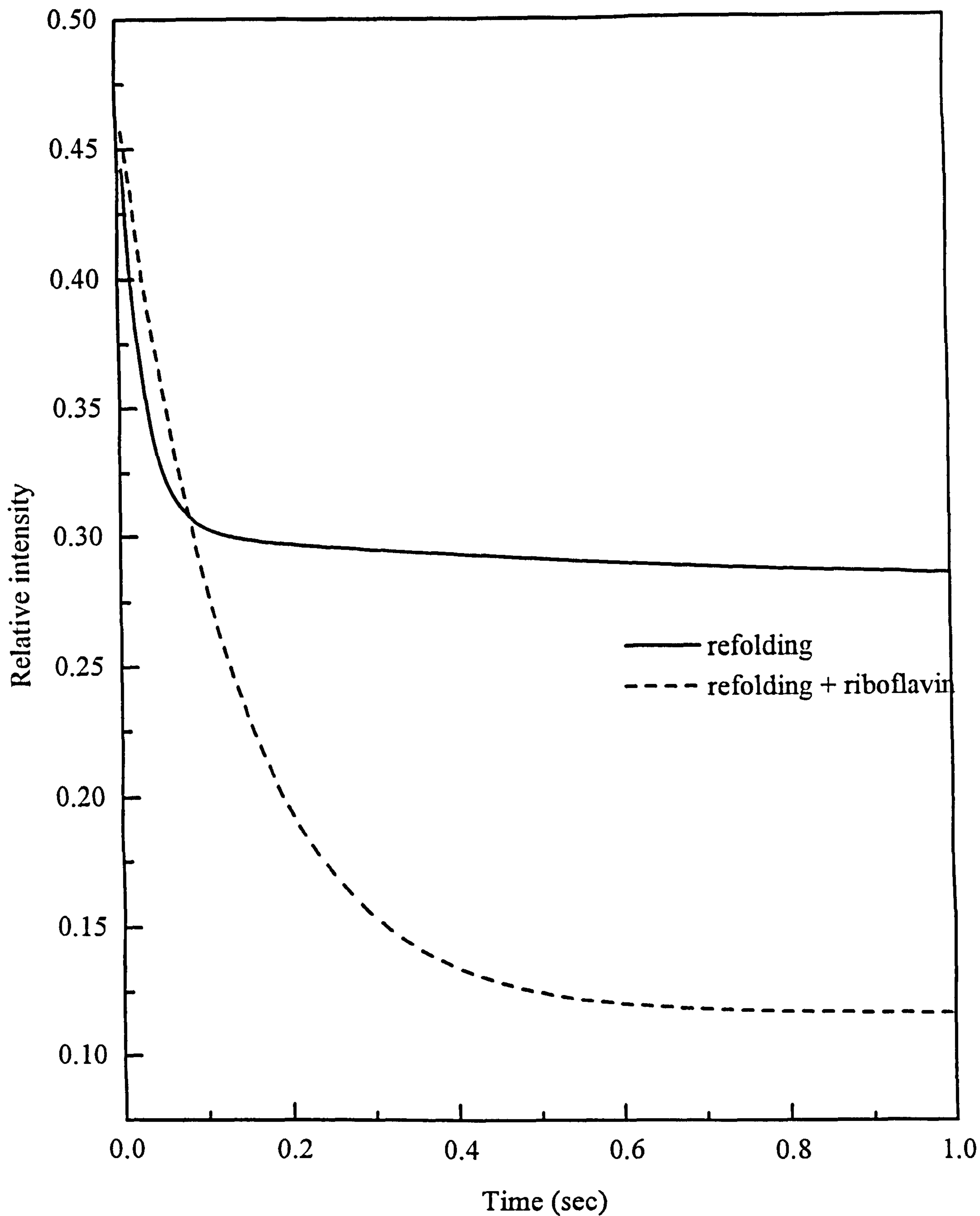


Figure 4.8a Fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence and absence of riboflavin (kinetic fits)

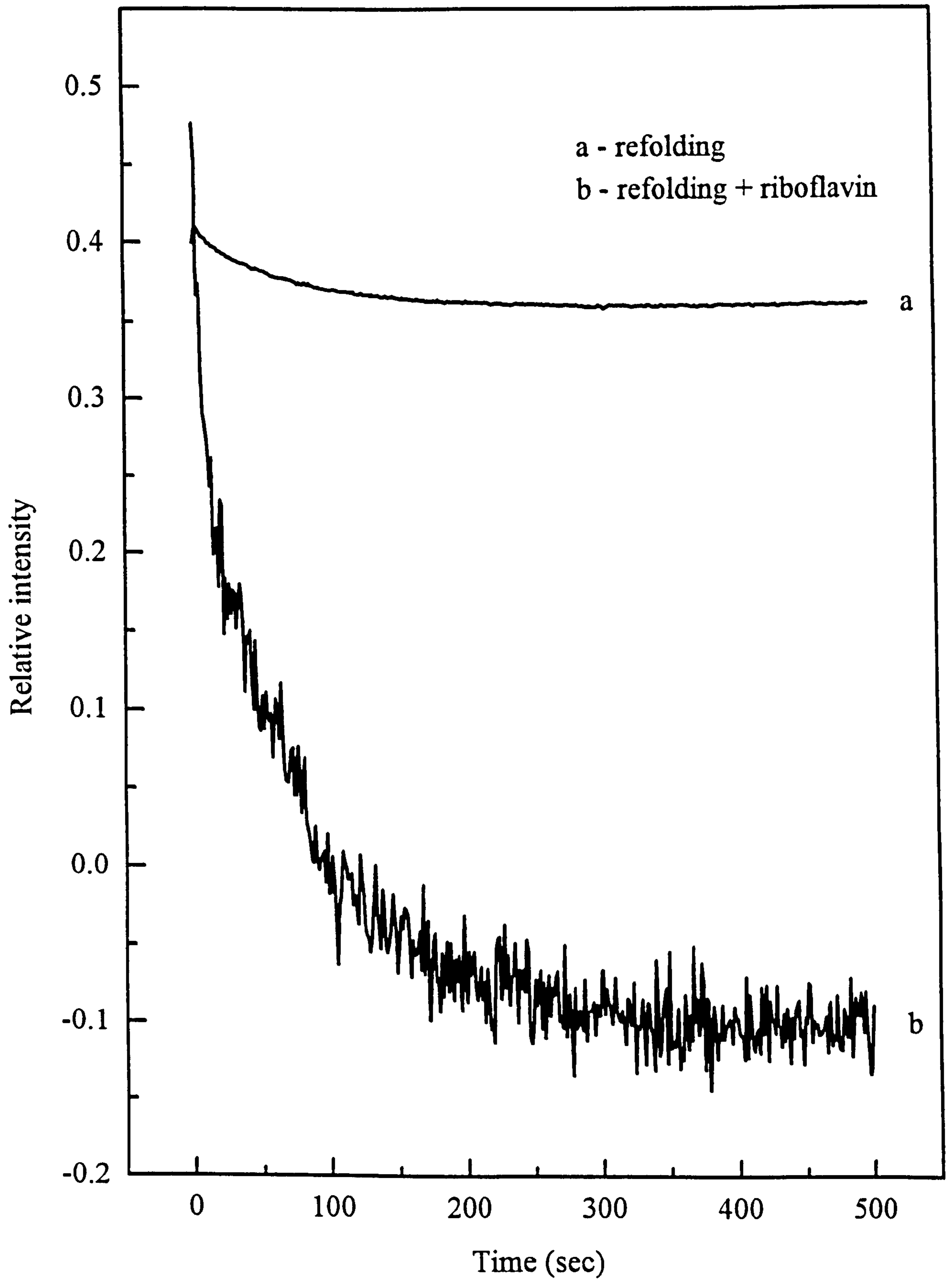


Figure 4.9 Fluorescence emission at 350 nm following dilution of Gdn HCl, in the presence and absence of riboflavin

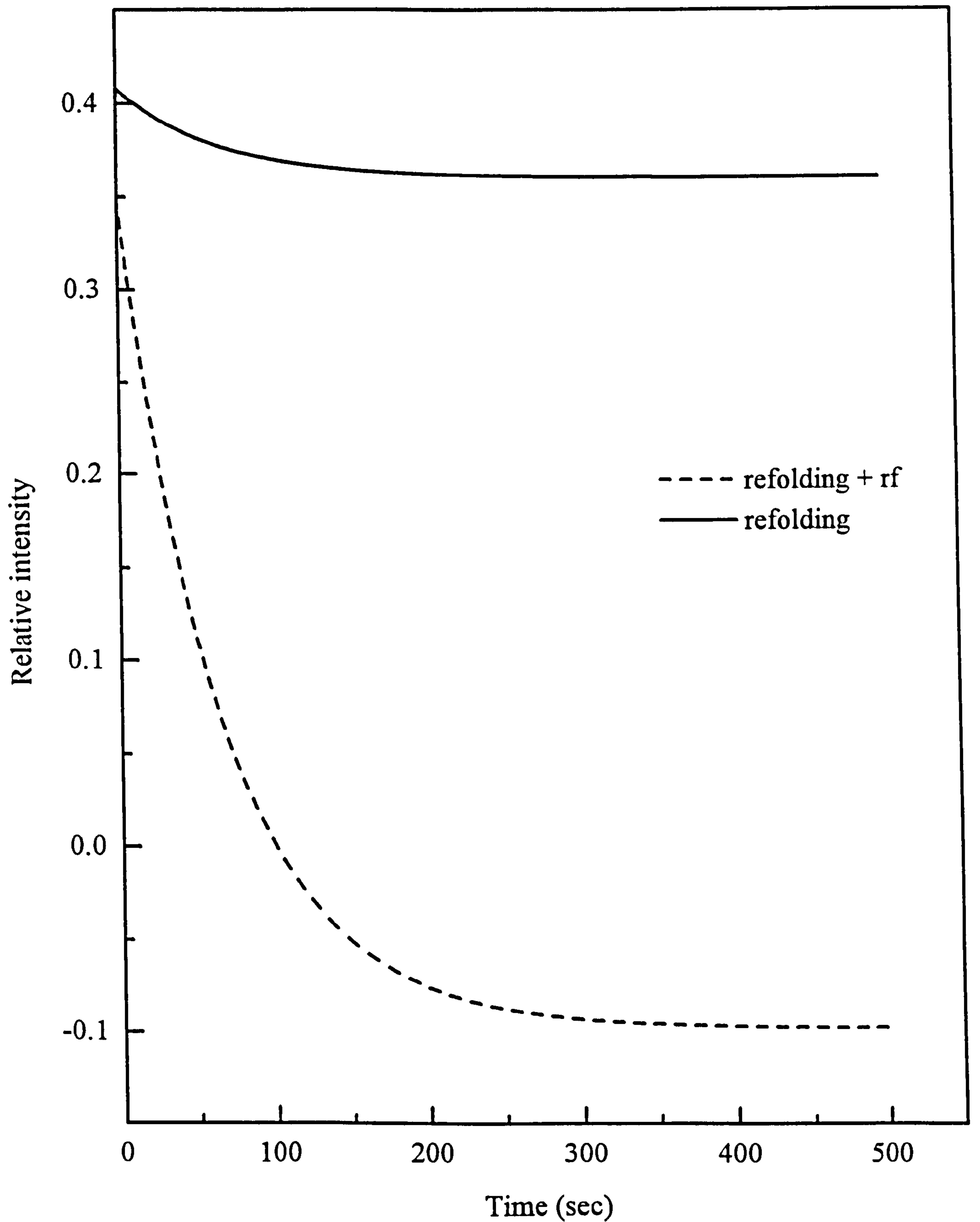


Figure 4.9a Fluorescence emission at 350nm following dilution of Gdn HCl, in the presence and absence of riboflavin (kinetic fits)

at 520 nm upon the addition of native and denatured (refolding) RfBP (Fig. 4.10). The binding of riboflavin by the native protein is very rapid ($t_{1/2} = 3.5$ msec). The regain of binding ability in the refolding protein was found to have similar kinetics to the fast phase found in tertiary structure acquisition ($t_{1/2} = 30$ msec), indicating that the binding pocket is formed during this stage.

4.3.3 ANS binding

The binding of 20 μ M ANS to apo-RfBP (8 μ M), resulted in a 20 fold increase in ANS fluorescence (Fig. 4.11). The intrinsic fluorescence of RfBP was quenched by approximately 50%, and the λ_{\max} slightly blue shifted, from 346 to 343 nm (Fig. 4.12). An equivalent enhancement of protein fluorescence quench and shift was found when the experiment was repeated with the holo-protein (Fig. 4.13) and the enhancement in ANS was identical to that found with the apo-protein. The apo-RfBP/ANS complex was able to bind, and quench the fluorescence of, riboflavin (Fig. 4.14). Although the denatured RfBP was unable to bind ANS, the ability to bind ANS was regained (as judged by the increase in ANS fluorescence) by the refolding protein within the dead-time of the instrument, i.e. before the regain of riboflavin binding ability (< 2 msec). The presence of ANS during refolding did not affect the ability of the refolded protein to bind riboflavin. The kinetics of refolding (Fig. 4.15), and riboflavin binding (Fig. 4.16), as measured on the stopped-flow instrument, were not affected by the presence of ANS.

4.4 Discussion

In spite of having nine disulphide bridges, RfBP appears to lose all detectable secondary

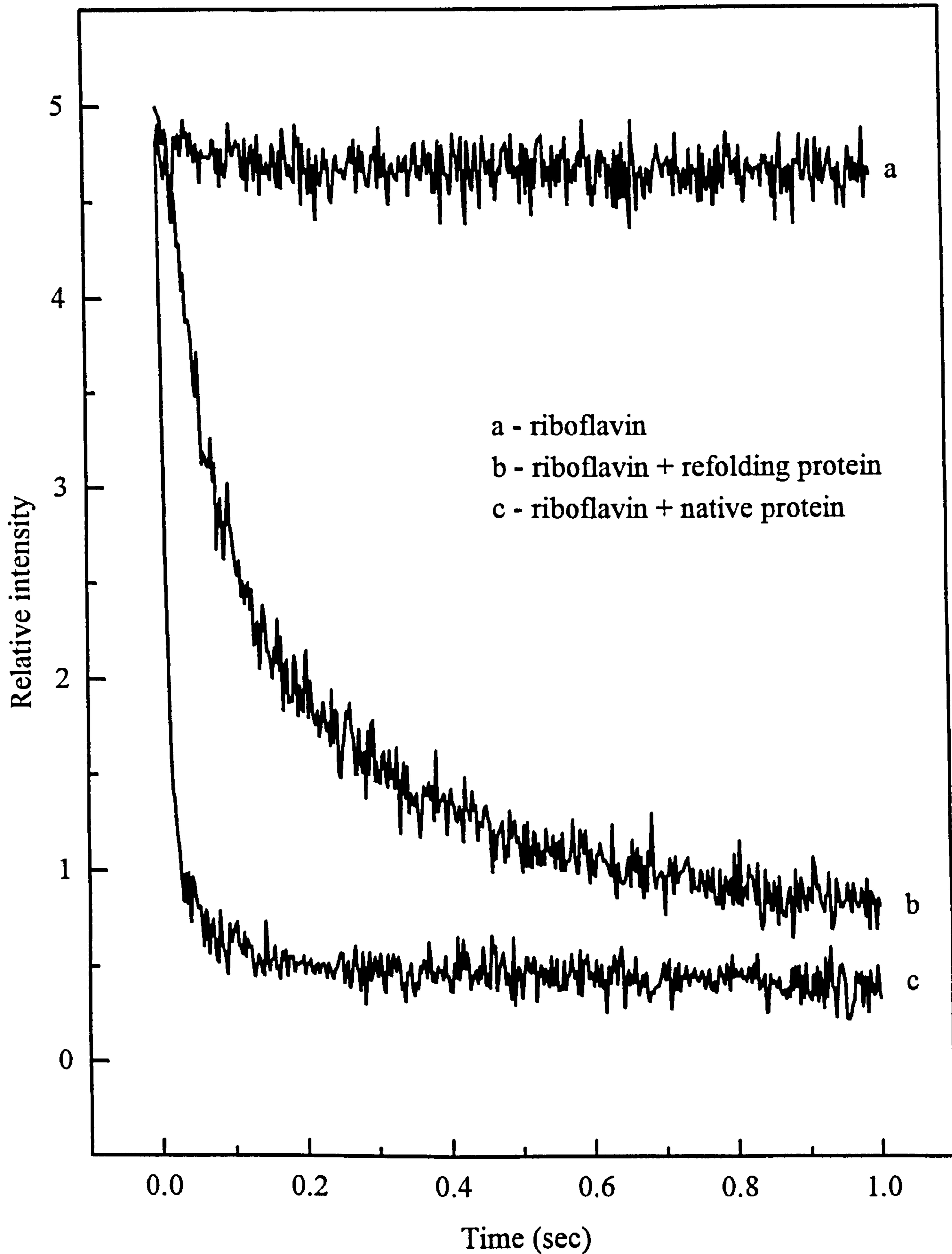


Figure 4.10 Riboflavin fluorescence quenching by native and refolding RfBP - changes in emission at 520 nm

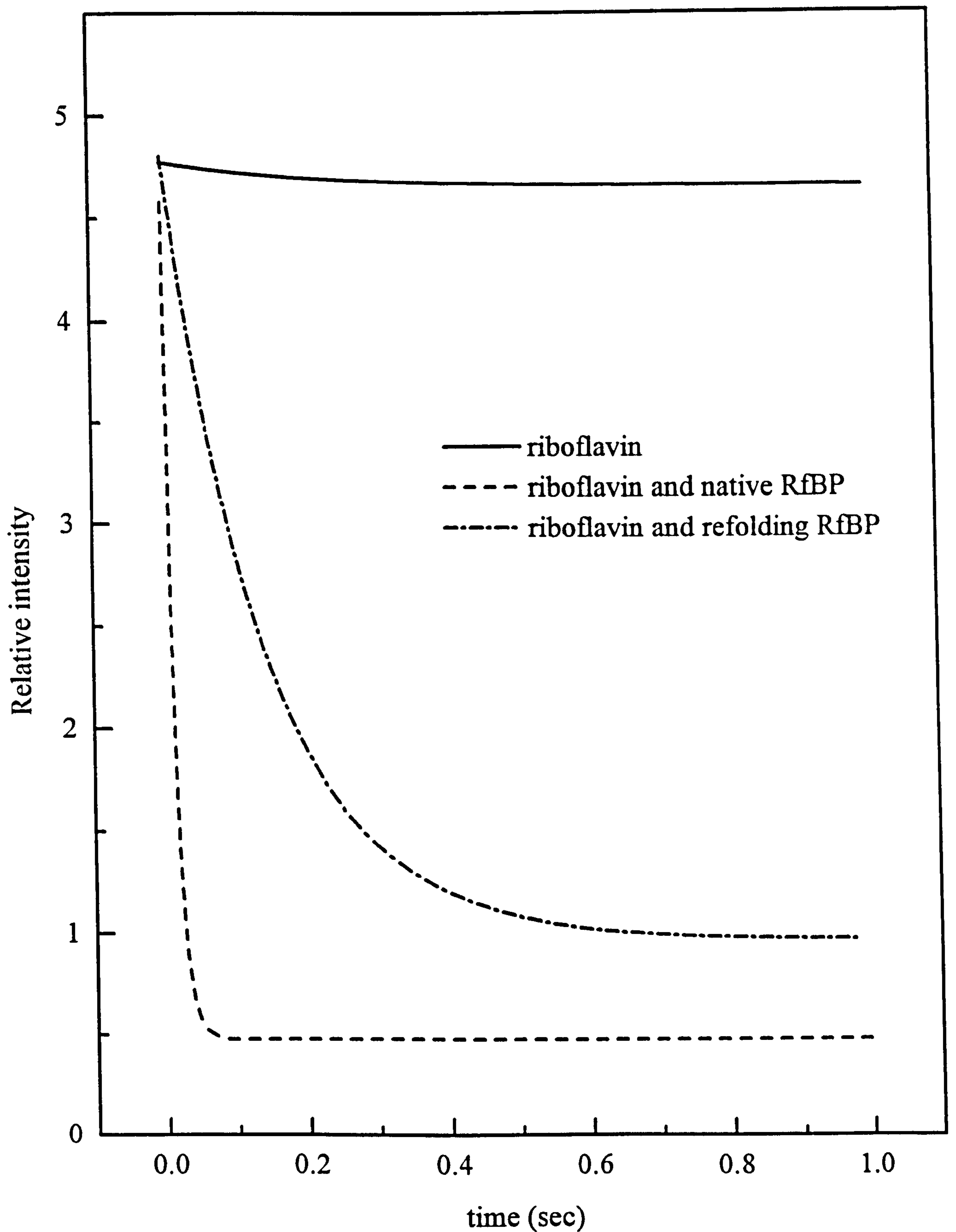


Figure 4.10a Riboflavin fluorescence quenching by native and refolding RfBP - changes in emission at 520nm (kinetic fits shown)

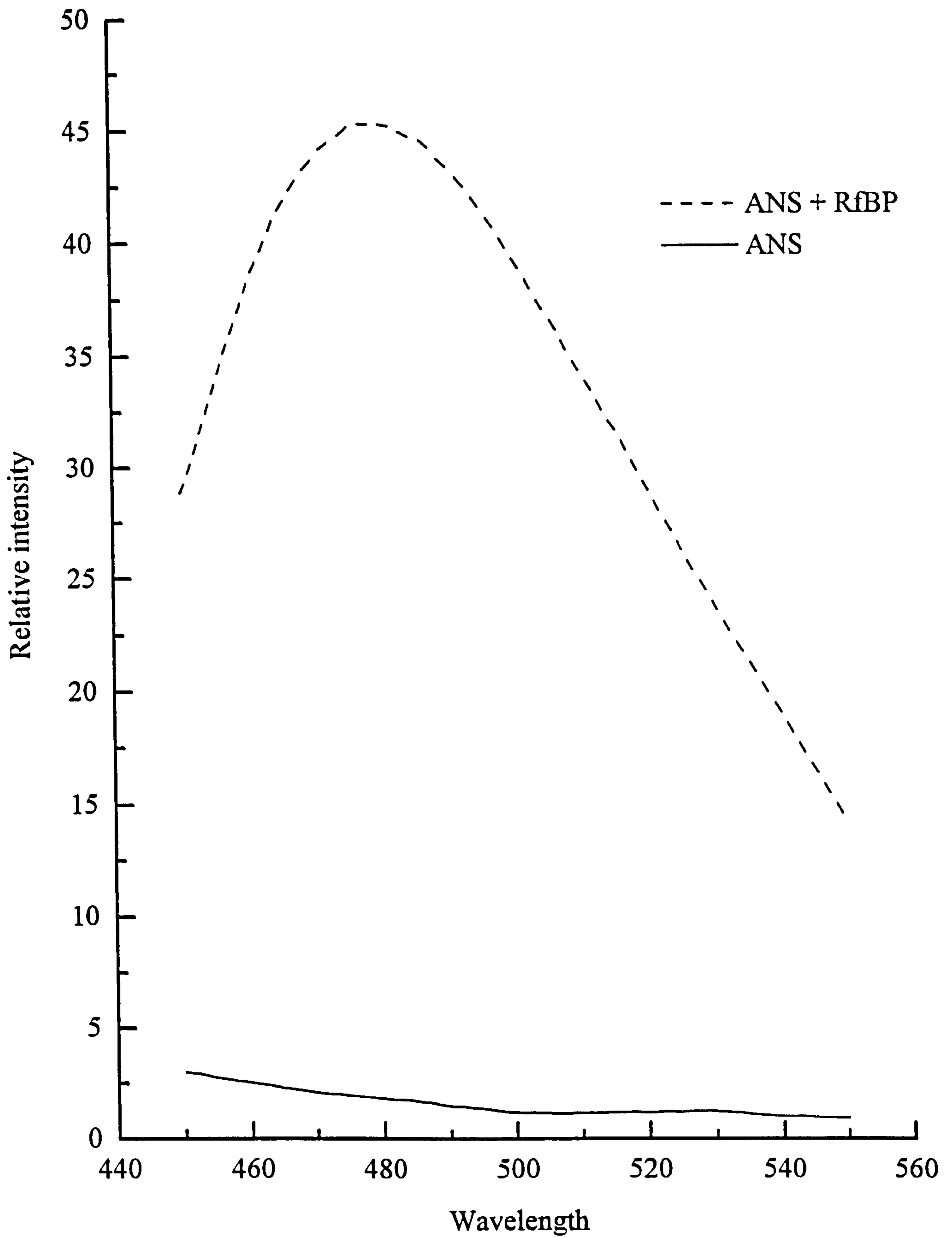


Figure 4.11 The fluorescence spectra of ANS in the presence and absence of apo-RfBP

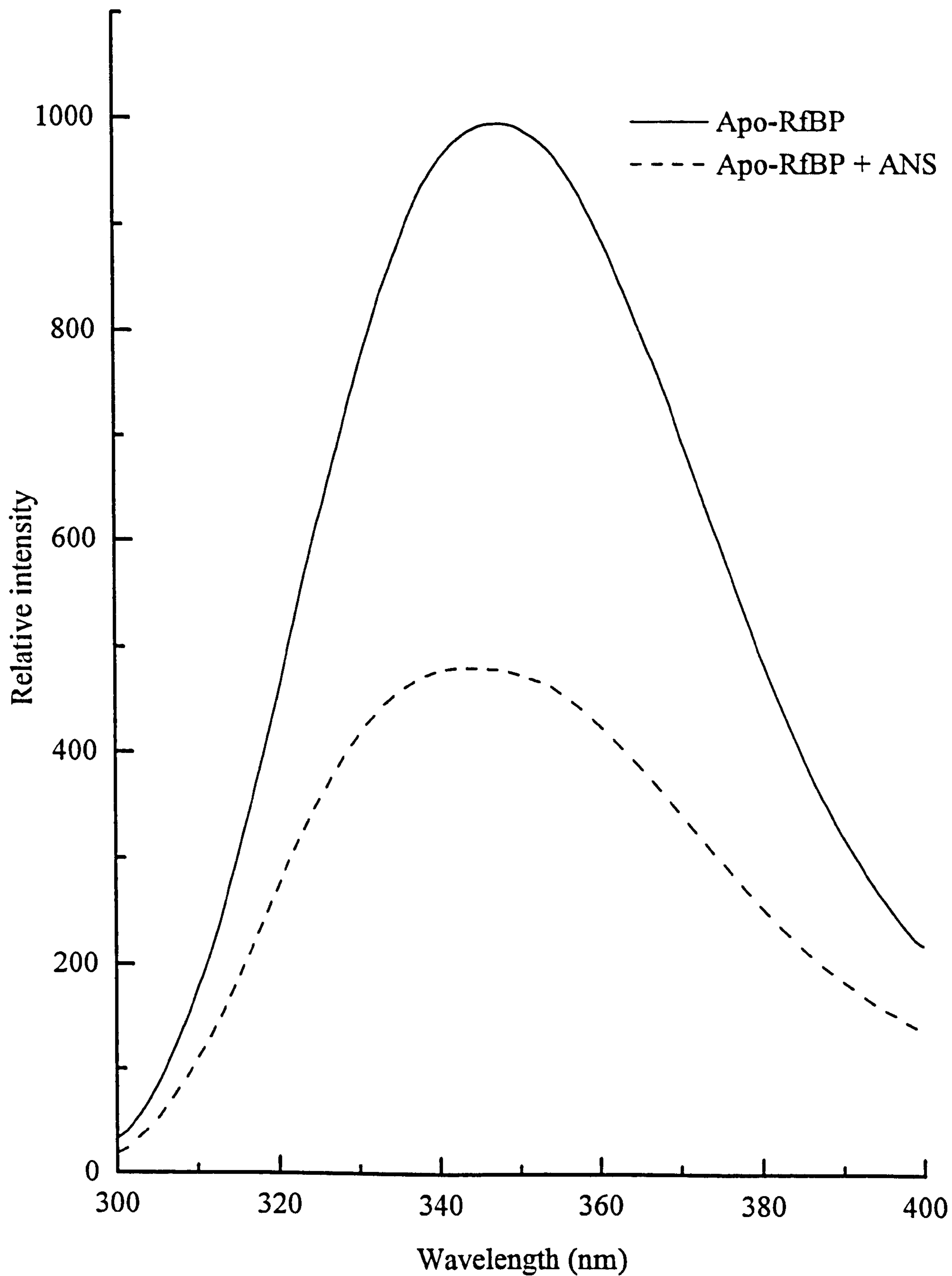


Figure 4.12 The fluorescence spectra of apo-RfBP in the presence and absence of ANS

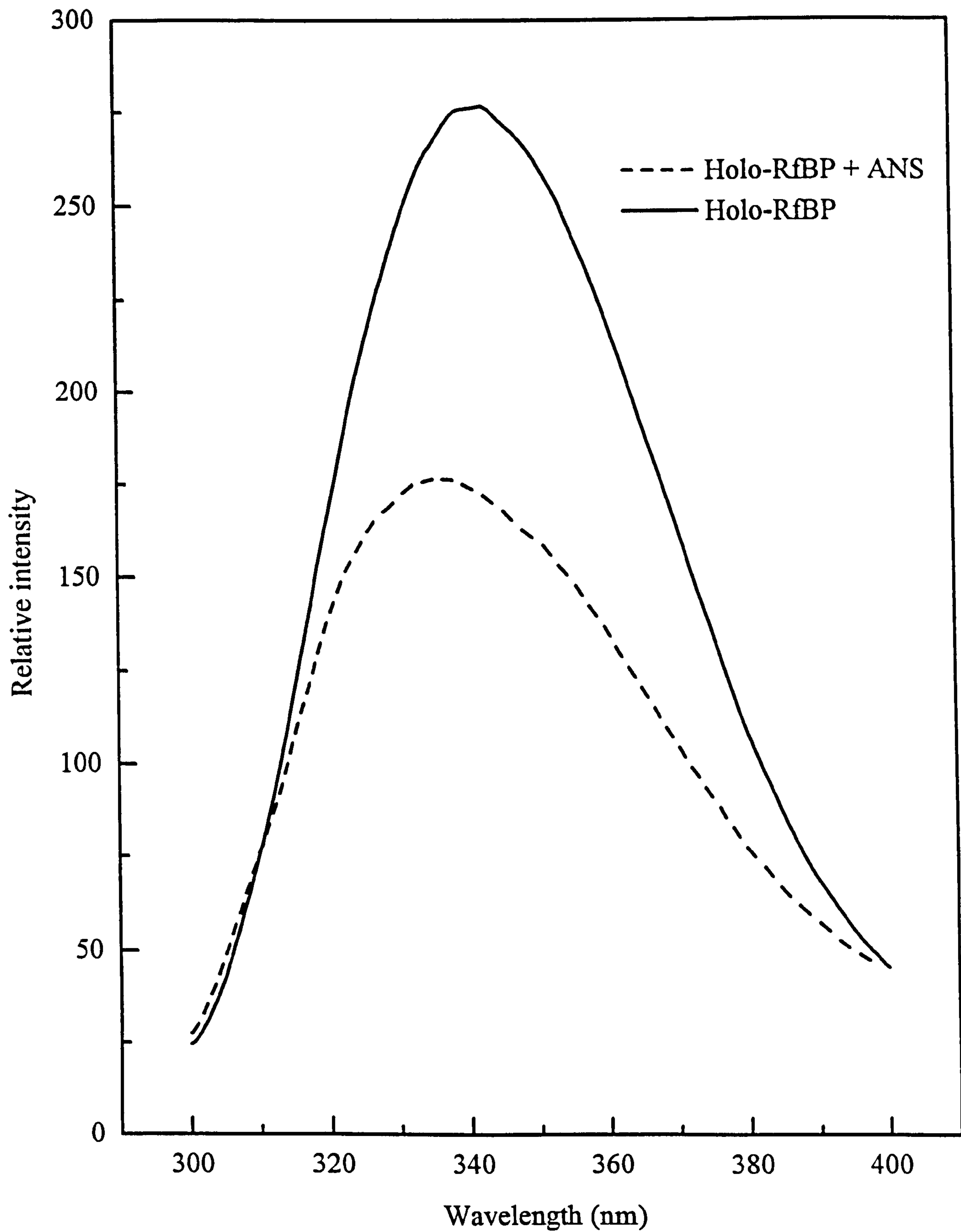


Figure 4.13 The fluorescence spectra of holo-RfBP in the presence and absence of ANS

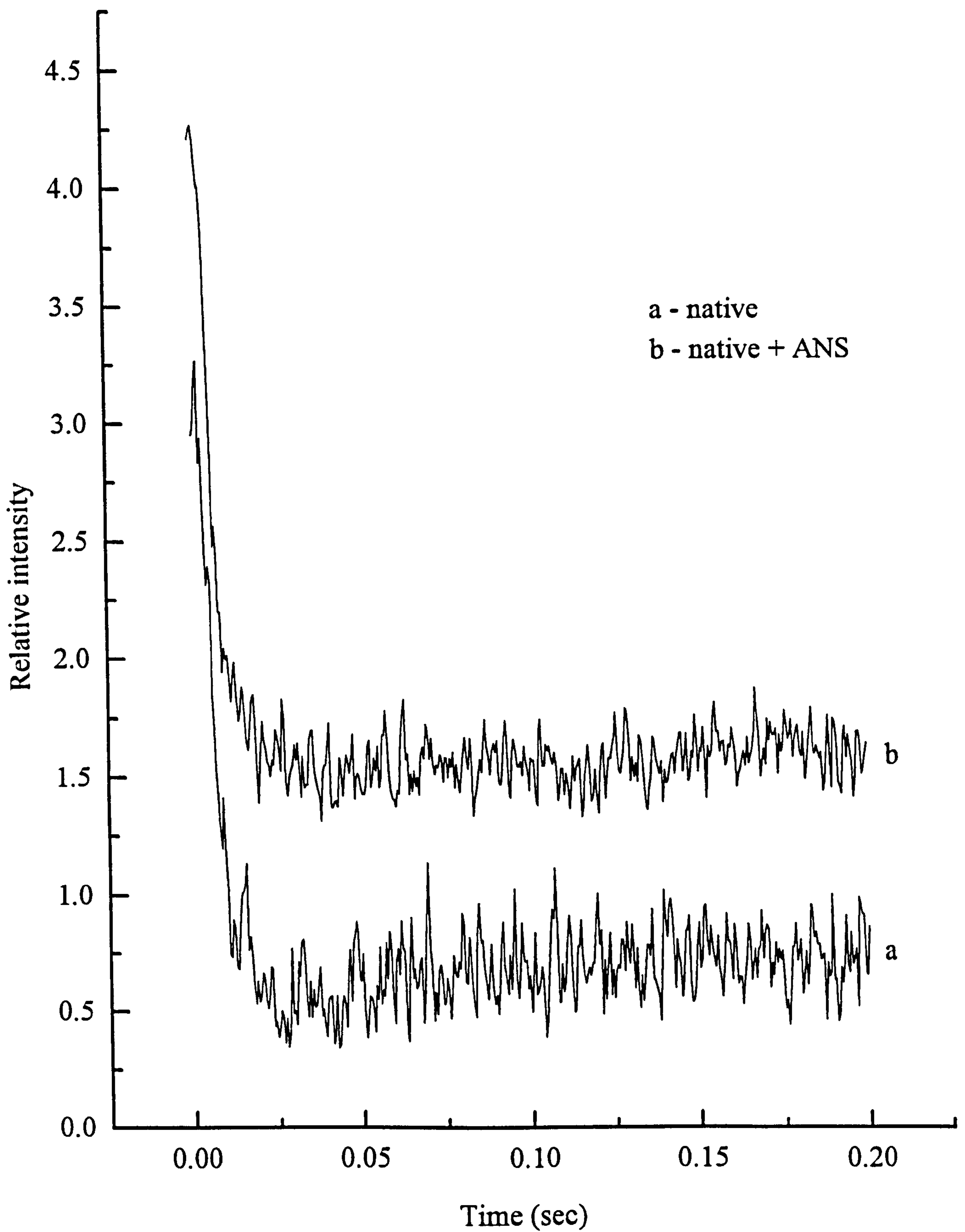


Figure 4.14 The quenching of riboflavin fluorescence by RfBP in the presence and absence of ANS

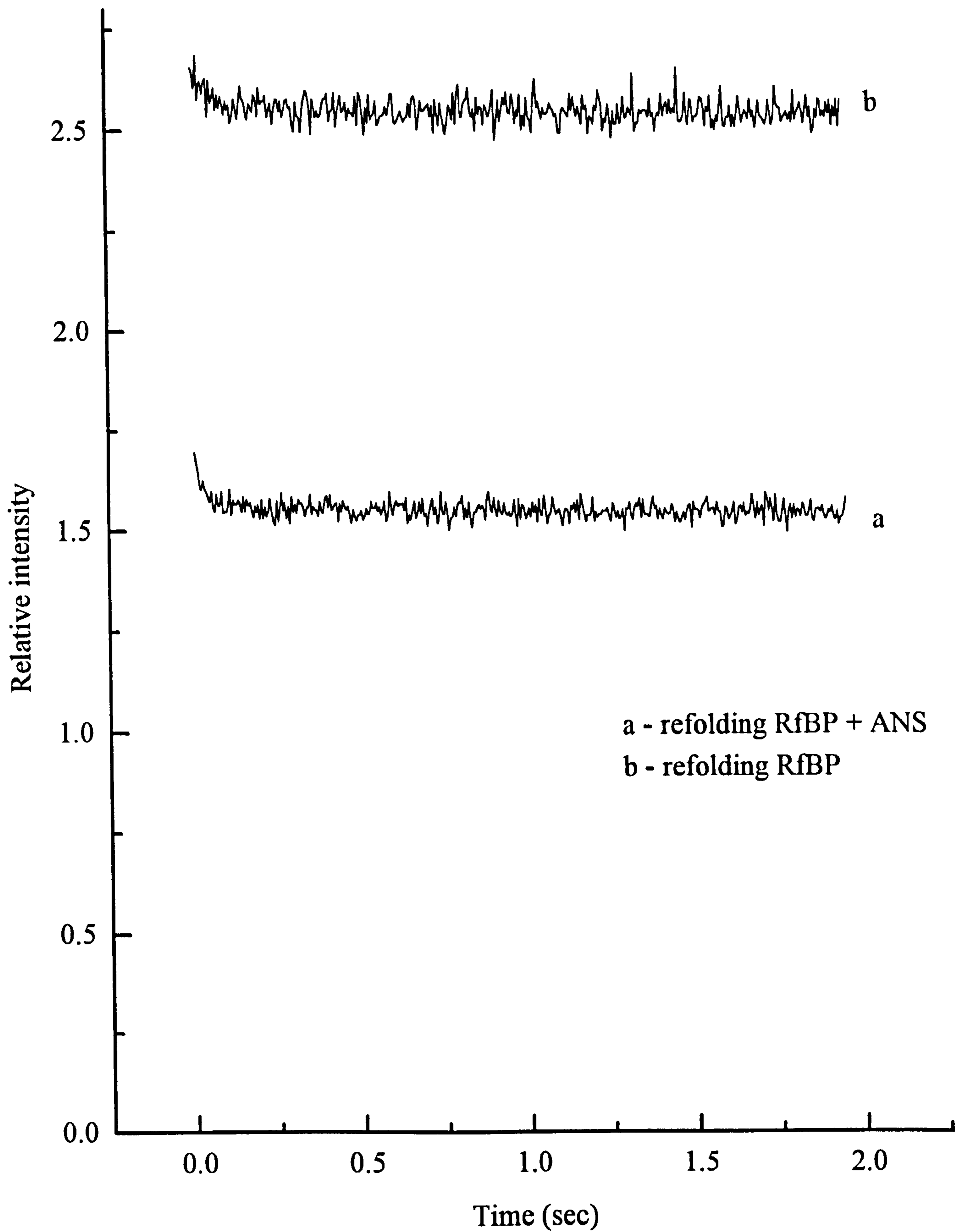


Figure 4.15 Changes in fluorescence at 350nm following dilution of Gdn HCl, in the presence and absence of ANS

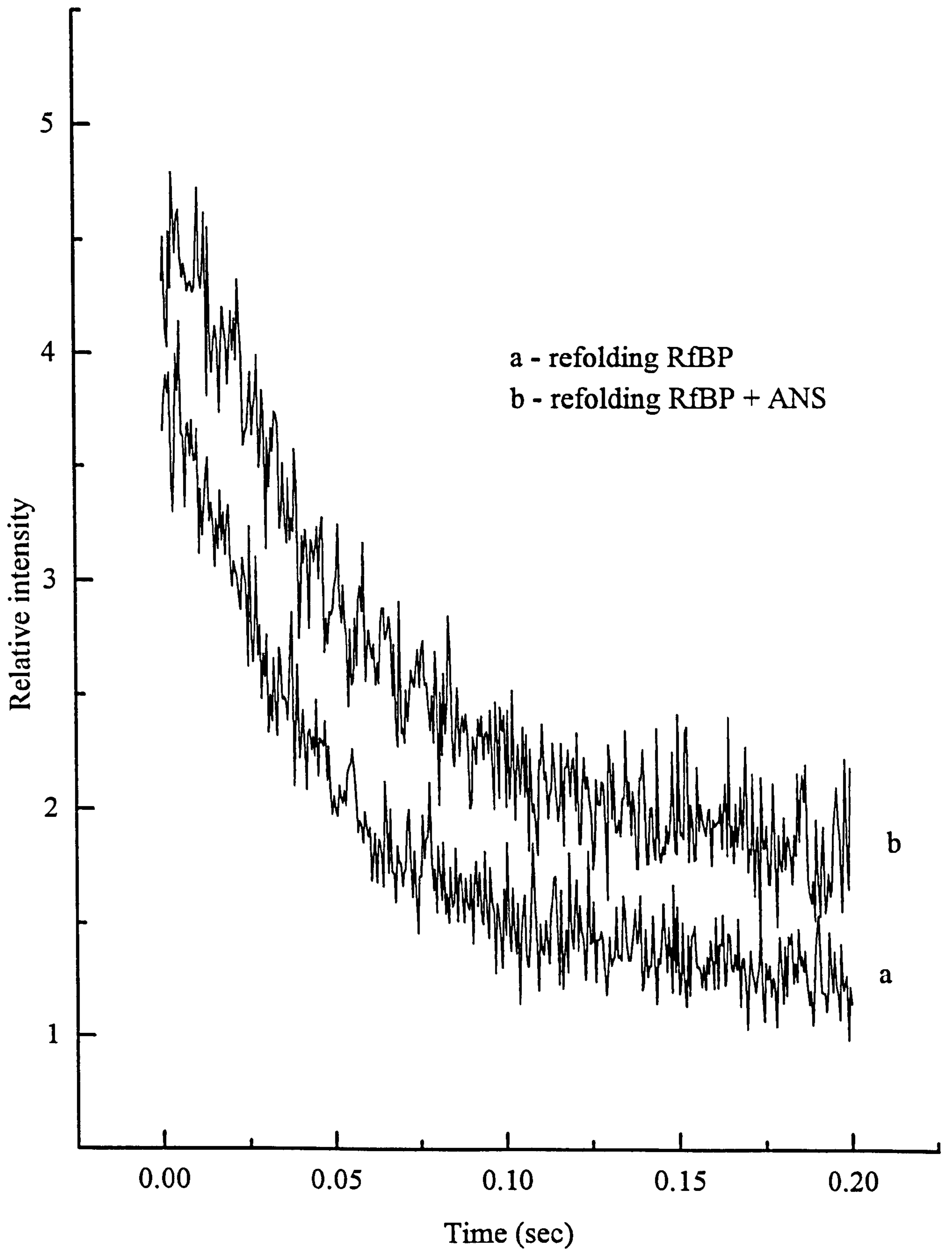


Figure 4.16 Regain of riboflavin binding ability in the presence and absence of ANS - fluorescence changes at 520 nm following the dilution of Gdn HCl

Table 4.4 The regain of tertiary structure in refolding RfBP

	V. fast	Fast	Slow	V. slow
Half- time ($t_{1/2}$)	< 1 msec	30 msec	46 s	60 min
% of total change	80	10	5	5

and tertiary structure after incubation in 6 M Gdn HCl. This is analogous to the behaviour of another disulphide bonded protein, lysozyme (Dobson *et al.*, 1994). The tertiary structure of RfBP was largely resistant to acidic pH, probably as a result of the stability conferred on the protein by the extensive disulphide bonding.

As long as the nine disulphide bonds remain intact, denatured RfBP was found to refold completely and very quickly after the removal of the denaturing agent, without the need for any other additional agents such as chaperone proteins. From the stopped-flow and steady state data, it is apparent that the refolding process occurs in several stages. The results show that all of the native secondary structure and a large proportion of the native tertiary structure are formed very quickly after the initiation of refolding, within the dead-times of the respective instrument modes. This is analogous to other systems studied *e.g.* lysozyme, cytochrome *c* (Dobson *et al.*, 1994; Elöve *et al.*, 1992). This could indicate that the protein structure formed during the very fast phase was similar to a molten globule, a compact, condensed molecule, with large elements of native secondary structure, but fluctuating tertiary structure. However, ANS binding studies did not follow the expected classic pattern of rapid binding, followed by slow release, an indicator of the formation and disappearance of a molten globule state (Ptitsyn, 1992). The pattern of ANS binding does, however, shed additional light on the refolding process. The native apo- and holo-proteins were both able to bind ANS, as is the case for a number of other proteins in their native state *e.g.* glutamate dehydrogenase and creatine kinase (Dodd and Radda, 1969; McLaughlin, 1974). During refolding, the protein regained the ability to bind ANS more quickly than the ability to bind riboflavin. It has been suggested that the presence of ANS from the start of

refolding can perturb the folding process, leading to the formation of ANS dependent kinetics and intermediate states (Engelhard and Evans, 1995). However the kinetics of the refolding reaction of RfBP as monitored by changes in protein fluorescence were not affected by the presence of ANS, nor was the riboflavin binding ability of the refolded RfBP. ANS tends to bind to clustered hydrophobic residues, so it might be expected that the hydrophobic riboflavin binding site could be a candidate for an ANS binding site. However, as the holo-protein can bind ANS, in a similar manner to the apo-protein, it is clear that the ANS is binding at a site, or sites, distinct from the riboflavin binding pocket. It is perhaps not surprising that refolding RfBP regains the ability to bind ANS long before riboflavin binding ability. The riboflavin binding site (Fig. 3.5) is thought to be a structured hydrophobic pocket, which binds riboflavin very tightly, indicating strong specific interactions between RfBP and riboflavin. ANS, on the other hand, only requires a non-specific, non-structured hydrophobic environment to bind to. One or more areas possessing such an environment could easily be rapidly formed following the initiation of refolding.

The quenching of riboflavin fluorescence by the native apo-protein is very fast ($t_x = 3.5$ msec), which is consistent with association rate constants for enzymes and substrates (Fersht, 1985). The regain of riboflavin binding ability coincides with the second phase of fluorescence regain ($t_x = 30$ msec), indicating that the refolding protein has assumed near native tertiary structure at this point. The third (slow) phase presumably reflects further small changes in the tertiary structure. The final regain of native tertiary structure can take up to two hours. This slow phase could reflect the *cis/trans* isomerisation of X-Pro bonds (Lang *et al.*, 1987). To verify this, a high resolution

structure of the protein would be needed, to determine the conformation of the 8 proline residues. The enzyme peptidyl prolyl isomerase could also be tested as a catalyst for this step in the folding of RfBP (Schönbrunner *et al.*, 1991).

It would appear that the presence of riboflavin during the refolding process somehow impeded the final acquisition of tertiary structure, albeit very slightly, and this had no effect on the binding of riboflavin. It could be that the binding pocket, or the riboflavin binding element, is formed relatively early in the refolding process, and the binding of riboflavin at this stage places constraints on small adjustments in the local tertiary structure.

Chapter 5 Reduction and Reoxidation of RfBP

5.1 Introduction

One of the most important factors involved in maintaining the specific three dimensional structure of a protein is the disulphide bond. This covalent link, formed by the oxidation of 2 cysteine side chains to form a cystine residue, adds greatly to the stability of a structure (Creighton, 1993).

The formation of disulphide bonds also has implications for protein folding. In Chapter 4, the refolding of the unfolded, oxidised RfBP was studied, *i.e.* the disulphide bonds were intact. Even though the molecule had all the characteristics of a random coil, as ascertained by CD (no discernable secondary structure), the existence of 9 covalent links in a molecule must limit the number of possible conformations accessible to it. It has also been demonstrated that the formation of disulphide bonds can occur co-translationally (Bergman and Kuehl, 1979; Peters and Davidson, 1982), and as such, must place considerable restrictions of the flexibility and possible conformations of the nascent polypeptide chain, again restricting the structural possibilities open to it.

Therefore, although valuable insights into the mechanism of folding can be gained from the study of refolding of oxidised molecules, it is important to study the formation of disulphide bonds during the folding process, in order to gain appropriate insights into the folding process *in vivo*.

5.1.2 Disulphide bonds in RfBP

RfBP is extensively disulphide bonded, containing 9 disulphide bridges (Fig. 1.1). It is undoubtedly these bonds which give RfBP its great stability - solutions of the protein can be boiled for 30 minutes (Rhodes *et al.* 1959), with no loss of activity and the protein does not denature until the temperature has reached 130°C (White and Merrill, 1988). All nine disulphide bonds appear to be of equal reactivity toward reducing agents: however the holoprotein appears to be more resistant to reduction than the apoprotein (Allen *et al.*, 1992). It has been proposed that destroying one of the disulphide bonds will disrupt riboflavin binding (Kozik, 1982a). This disulphide is not protected by riboflavin, and so is thought to lie outside the binding pocket.

5.1.3 Protein disulphide isomerase

Protein disulphide isomerase (PDI) (EC 5.3.4.1) was first described as a "general and non-specific catalyst for disulphide interchange in proteins containing disulphide bonds" over thirty years ago. It was discovered by Anfinsen and his co-workers (Goldberger *et al.*, 1963), who had postulated the existence of additional factors assisting *in vivo* folding when they demonstrated that the amino acid sequence contained all the information necessary for the folding of a polypeptide chain into a mature protein. Some structural characterisation of PDI was also carried out at the time, but the enzyme was largely ignored until the 1980's. Since then, however, a number of studies have confirmed the location, action and role of PDI in the cell, thus verifying that PDI is responsible for the formation/isomerisation of disulphide bonds during the folding of proteins (Freedman *et al.*, 1989; Freedman, 1992).

5.1.3.1 Structure of PDI

PDI is found in the lumen of the endoplasmic reticulum (ER)(Freedman, 1989). It is very abundant, accounting for approximately 0.4% of the total cellular protein (Hillson *et al.*, 1984). Its concentration may be in the millimolar range (Gilbert, 1989). PDI is active as a homodimer (M_r 110000), with no interchain disulphide bonds (Freedman *et al.*, 1984). The major parts of the sequence are widely conserved across eukaryotes, and it has a prokaryotic counterpart in DsbA, which is found in the periplasm of bacteria; plant homologues have also been found (Freedman *et al.*, 1994). The monomer of PDI consists of 6 domains (a,a',b,b',c and e). A and a' are 47% identical, and have been shown to be homologous to thioredoxin, a small ubiquitous protein, involved in many cytoplasmic redox reactions (Freedman *et al.*, 1994). The active site of PDI is structurally homologous to that of thioredoxin, and consists of a short loop containing the sequence -CGHC-. The two Cys residues have been clearly implicated as the redox active sites of catalysis, by mutagenesis and chemical studies. The two domains of PDI can function independently (Freedman *et al.* 1994).

The exact mode of action of the enzyme in its disulphide formation/isomerisation role has not yet been clarified; such studies are hampered by the size of the PDI molecule, and of the substrate, and the complexity of the reaction catalysed (Freedman *et al.*, 1994). It is thought that PDI generates disulphide bonds that "permit the most stable folded conformation of the system as a whole at the applied thiol/disulphide redox potential" (Freedman, 1992). PDI has an extremely wide substrate range (potentially all proteins containing disulphide bonds).

5.1.3.2 Other roles for PDI

PDI has been shown to be a multifunctional enzyme, and has been found to be present as a subunit of other complex multienzyme systems. Prolyl-4-hydroxylase catalyses the formation of 4-hydroxyprolyl residues in nascent collagen-like polypeptides. The protein is a tetramer, with an $\alpha_2\beta_2$ subunit structure. It was known for some years that many cells contained an large excess of β -subunits, and that newly synthesised α subunits recruited β subunits from this pool - later studies showed that the β -subunits are in fact PDI, the pool corresponding to the free PDI of the lumen (Koivu *et al.*, 1987). The role of PDI in this instance appears to be to ensure the solubility and integrity of the native complex; when expressed in the absence of PDI in a cell-free expression system, the α -subunits aggregate. The Cys residues implicated in disulphide formation/isomerisation are not involved in this role of the enzyme (Vuori *et al.*, 1992).

PDI is also the β -subunit of triacylglycerol transfer protein. This protein incorporates lipids into newly synthesised core lipoproteins in the ER. Similarly to P4H, the protein exhibits no PDI-like activity, but upon dissociation of the dimer, the α -subunits aggregate, and soluble PDI activity returns (Wetterau *et al.*, 1990).

A number of other functions have been ascribed to PDI. It exhibits affinity for thyroid hormones, and has been studied as a thyroid-binding protein. It has also been shown to have an affinity for peptides, including glycosylatable peptides (Noiva and Lennarz, 1992). It has also been suggested that PDI may act in a general chaperone role, assisting folding and preventing aggregation within the ER (Noiva and Lennarz, 1992).

5.1.3.3 *In vitro* use of PDI

A number of studies have investigated the effects of PDI on the refolding of reduced unfolded proteins (Lyles and Gilbert, 1991; Lille *et al.*, 1994; Wunderlich *et al.*, 1995). Lille *et al.* (1994) used an antibody Fab fragment as a substrate. PDI has been implicated with the folding of Fab *in vivo*, since covalently linked complexes of PDI and nascent antibody light chains have been isolated from cells (Roth and Pierce, 1987). Lille *et al.* (1994) found that although PDI had no effect on the rate of refolding of the oxidised protein (i.e. no chaperone activity), the rate of reoxidation of the reduced protein was increased approximately 10-fold.

5.2 Methods

5.2.1 Reduction of RfBP

Apo-RfBP was reduced by the method of Kozik (1982a). All buffers and solutions contained 2.7 mM EDTA and were freshly purged with O₂-free nitrogen for 2 minutes to minimise random reoxidation by atmospheric oxygen, catalysed by trace metals. 1 ml of a 1 mg/ml RfBP solution was incubated overnight in 8 M urea and 1 mM DTT at room temperature.

The reduced RfBP was separated from excess urea and DTT by gel filtration on Sephadex G-25. The elution buffer (50 mM sodium phosphate, pH 7.8) contained 1 mM GSH, to prevent reoxidation of the reduced RfBP. The sulphhydryl content of reduced RfBP was determined spectrophotometrically by reaction with Nbs₂ (Ellman, 1959) using a sample which had been rapidly gel filtered in the absence of GSH. The CD and fluorescence spectra of the reduced RfBP were recorded.

5.2.2 Estimation of free sulphhydryl groups

Nbs₂, (Ellman's reagent) is widely used for assaying free thiol groups. It reacts with thiol groups to liberate one mole of 2-nitro-5-thiobenzoate per mole of thiol groups. This molecule has an intense yellow colour, and its formation can be monitored spectrophotometrically at 412 nm (Ellman, 1959).

5.2.3 Reoxidation of reduced RfBP

Reoxidation was initiated by the addition of GSSG to a final concentration of 0.1 mM. This ratio of [GSH]/[GSSG] (1/0.1) was found to be the optimum through reoxidation studies. The extent of regain of the native structure of RfBP was assessed by measuring the quenching of riboflavin fluorescence (as described in Section 2.7) of samples taken from the reoxidation mixtures, over periods up to five days.

5.2.4 The effect of PDI on the reoxidation of RfBP

When the effect of PDI was being studied, PDI was added to the solution of reduced RfBP in GSH immediately prior to the addition of GSSG. This order of addition was found to be essential. A series of reactions were set up, with increasing PDI:RfBP molar ratios, from 1:2 up to a maximum of 5:1. The reoxidation reactions were performed at 4°C. Control experiments were performed with equivalent molar ratios of BSA in place of PDI, to verify that the successful reoxidation did not arise from a general protein/protein interaction. Control experiments showed that the presence of PDI or BSA did not interfere with the measurement of riboflavin binding.

PDI was purified from bovine liver by the method of Freedman *et al.* (1995), and was generously supplied by Stephen McLaughlin, University of Kent.

5.3 Results

5.3.1 Reduction and reoxidation of apo-RfBP

After reduction of apo-RfBP in denaturing conditions (the urea partially unfolds the protein, allowing the DTT access to internal buried disulphide bonds), and gel filtration to remove excess DTT and urea, reaction with Nbs₂ revealed a sulphhydryl content of 20 ± 3 per molecule RfBP, in reasonable agreement with the value of 18 expected from the primary structure of the protein. The CD spectrum of the reduced protein was identical to that previously published (Allen *et al.*, 1992) (Fig. 5.1). The wavelength of fluorescence emission maximum was 349 nm (Fig. 5.2). Previous work had suggested that the emission maximum of the reduced species was 354 nm (Allen *et al.*, 1992). It is possible that the reduction method employed by Allen *et al.* (involving NaBH₄) may lead to more extensive denaturation of the reduced protein. The CONTIN secondary structure analysis program was applied to the CD spectrum of reduced RfBP (Provencher and Glöckner, 1981). It predicted an decrease in the level of α -helix, from 38 % to 24 %. From these studies it is clear that reduced RfBP has a lower content of secondary structure and more highly exposed tryptophan side chains than native RfBP, but still retains some folded structure.

The reduced RfBP was unable to bind riboflavin, as shown by the failure of the reduced RfBP to quench riboflavin fluorescence (Fig. 5.3). Control experiments showed that the

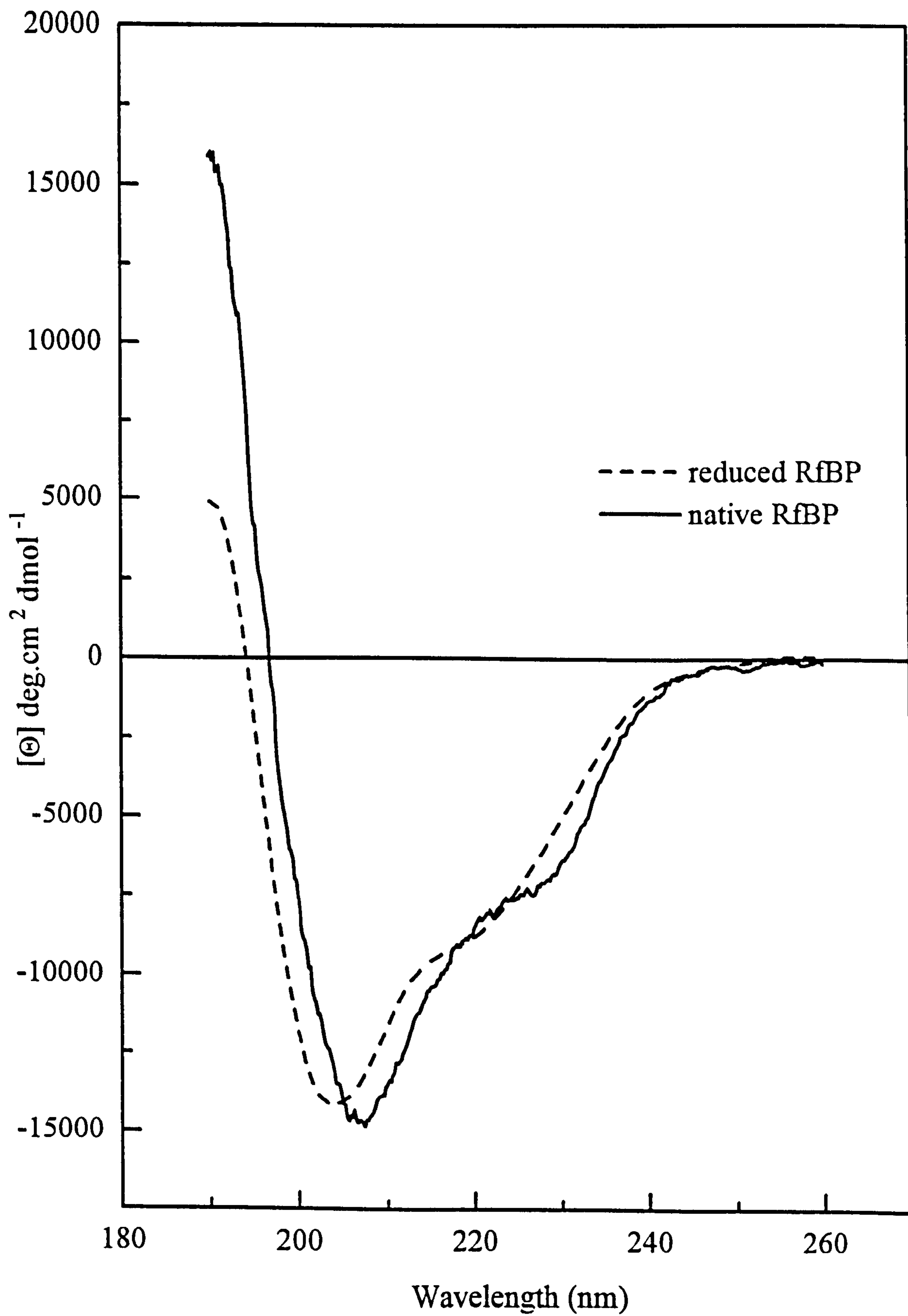


Figure 5.1 The far UV CD spectra of native and reduced RfBP

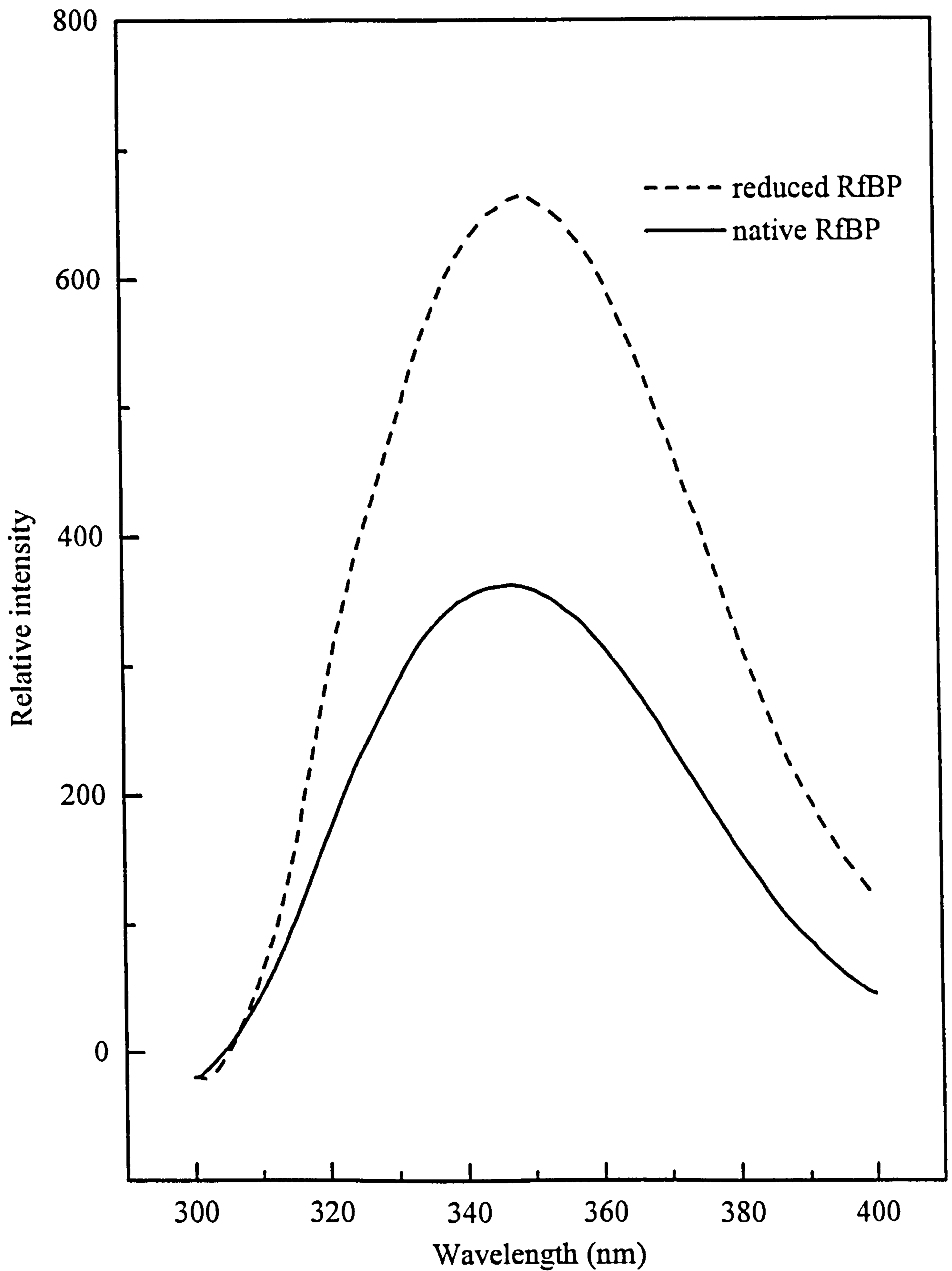


Figure 5.2 The fluorescence spectra of native and reduced RfBP

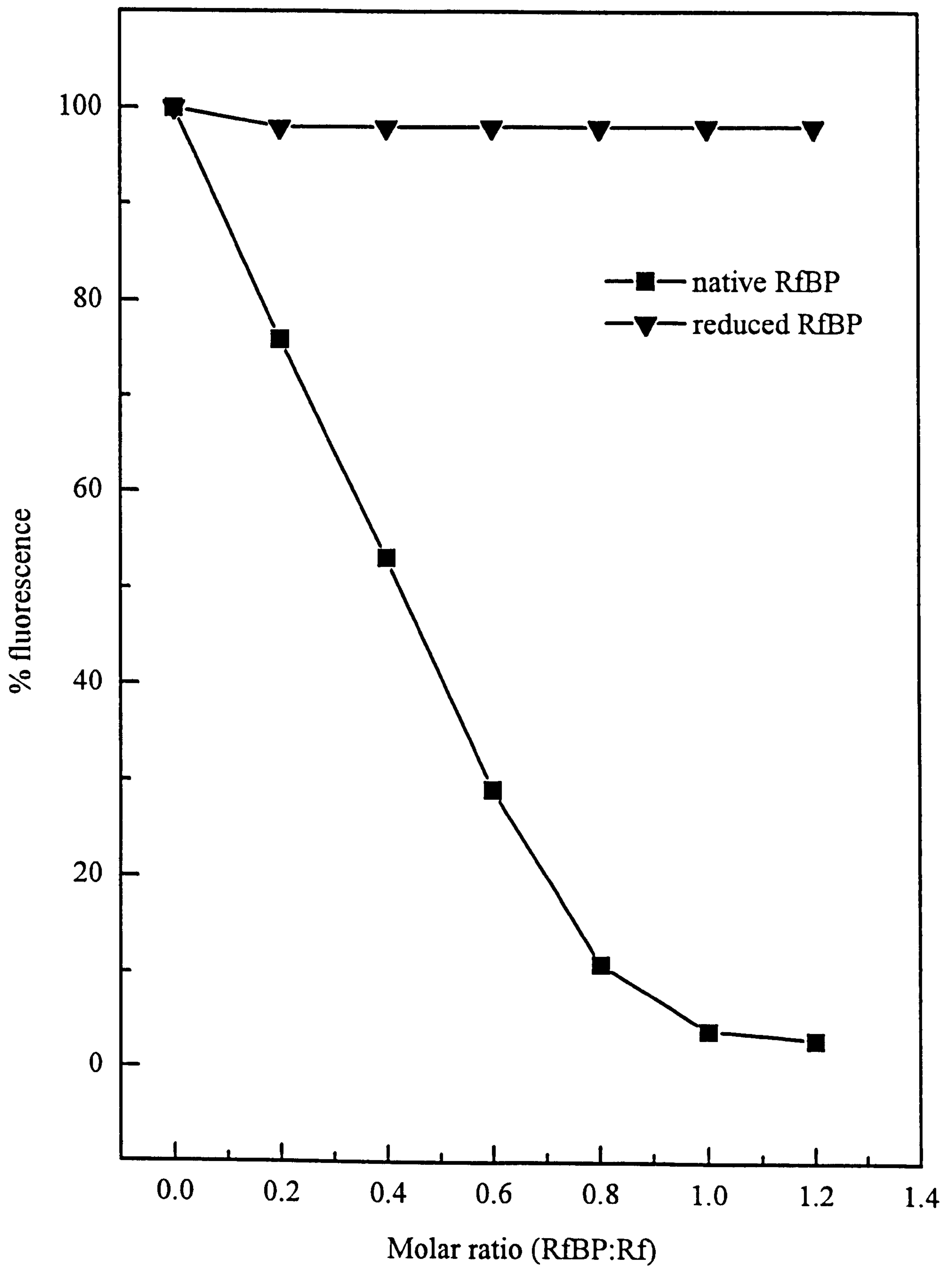


Figure 5.3 The quenching of riboflavin fluorescence by native and reduced RfBP

presence of GSH and or GSSG at the concentrations used in these studies had no effect on the binding properties of native oxidised RfBP (results not shown). Treatment of the reduced apo-RfBP with GSH/GSSG alone led to complete oxidation of the sulphhydryl groups, as shown by the lack of reaction with Nbs₂, after dialysis to remove excess GSH and GSSG. The overall structural properties of the reoxidised RfBP as measured by CD and fluorescence showed wide variations between material obtained in replicate experiments, presumably indicating that formation of a specific set of disulphide bonds had not occurred.

The regain of binding activity during reoxidation is expressed as a percentage of the quenching of riboflavin fluorescence observed with a control sample of native RfBP of similar concentration to the reoxidising RfBP (0.04 mg/ml) incubated at 4°C in the GSH/GSSG mixture for an identical period of time. Less than 5% quenching was observed with the reoxidised RfBP, indicating that no significant binding ability had been regained.

The effect of inclusion of PDI during the reoxidation was studied. It was found that reproducible data could only be obtained if all buffers were flushed with O₂-free nitrogen for 2 min immediately prior to the reoxidation experiments. It was also found that recovery of activity was higher at 4°C than at 20°C, and increased with increasing concentrations of PDI. Under the optimum conditions explored (i.e. a 5 fold molar ratio of PDI added to RfBP prior to addition of GSSG at 4°C) approximately 45% of the binding ability of native apo-RfBP could be regained (Fig. 5.4). In contrast, a maximum of 4.5% recovery was achieved with the GSH/GSSG redox system in the

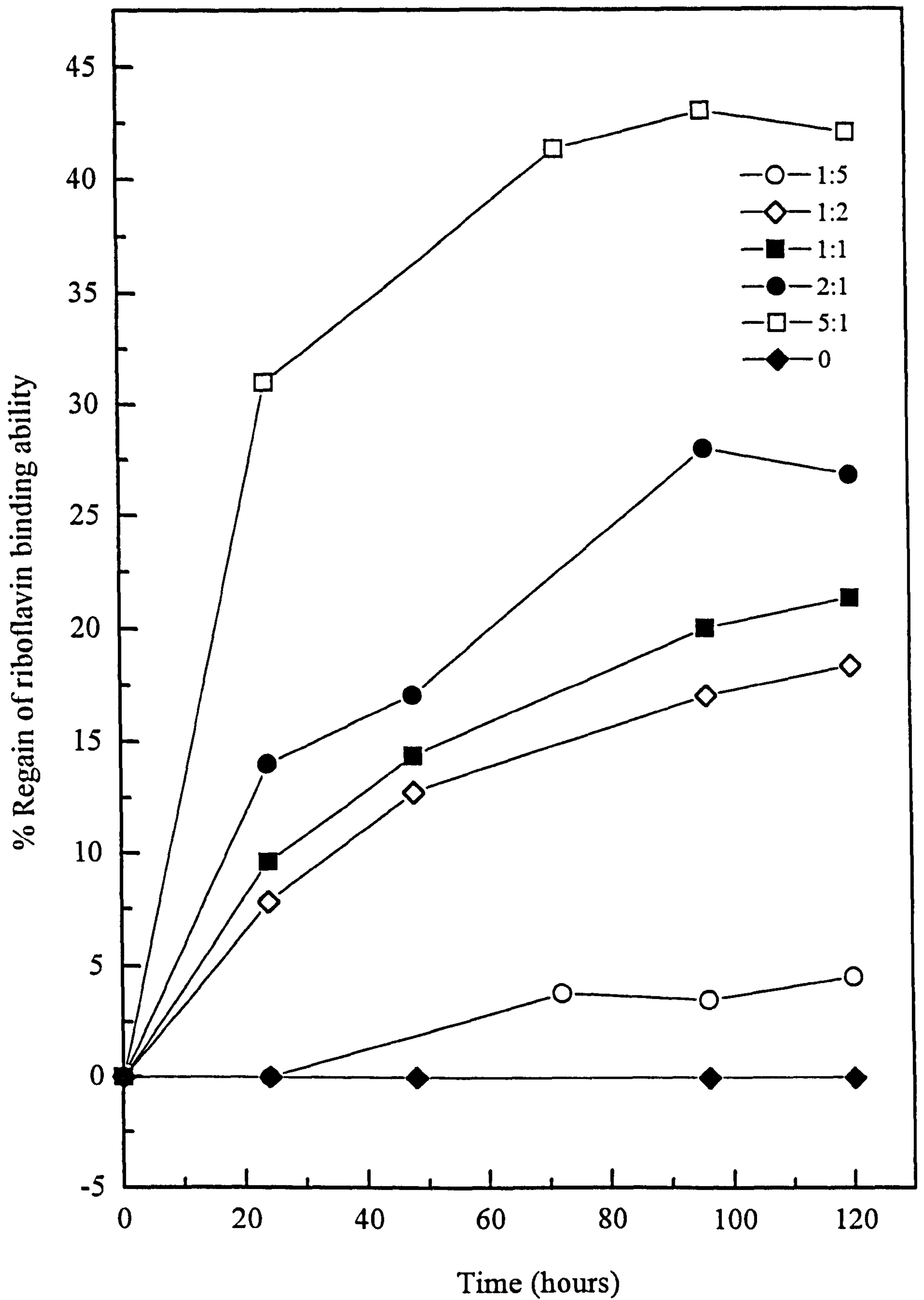


Figure 5.4 The effect of PDI on the regain of riboflavin binding ability (ratio = mol PDI: mol RfBP)

absence of PDI. At lower molar ratios of PDI:RfBP, lower degrees of recovery of activity were observed (Fig. 5.4). Addition of BSA to reoxidising RfBP in place of PDI yielded no active protein (Fig. 5.4), indicating that the effect of PDI is specific and does not represent a general chaperone-type action. In agreement with the studies of Lilie *et al.* (1994) on the refolding of reduced Fab, it was found that the effect of PDI is very dependent on the time of addition of PDI to the reoxidation mixture, with yields of reactivation being decreased substantially if the PDI is not present at the start of the reoxidation (i.e. if the PDI was added after the GSSG, used to initiate the reoxidation).

Because of the presence of large amounts of PDI, it was not possible to measure the structural properties of the reoxidised RfBP by spectroscopic methods.

5.4 Discussion

While the oxidised denatured protein would readily refold upon the removal of the denaturant, it was found that the reduced denatured molecule (disulphide bonds broken) was unable to refold spontaneously to the native state, on dilution of the denaturant, under oxidative conditions.

The extent of formation of the correct disulphide bonds during the reoxidation of reduced RfBP was clearly greatly enhanced by the presence of PDI. The use of PDI in reoxidation experiments increased the yield of correctly reoxidised RfBP by at least 10-fold to a value approaching 50%. These findings are similar to those reported by Lilie *et al.* (1994), who found that PDI, in molar concentrations comparable with or

greater than those of reduced Fab, increased successful reoxidation of the protein. Optimum regain of activity was obtained when the PDI:RfBP ratio was 5:1. Given that the concentration of PDI in the lumen of the endoplasmic reticulum is thought to be in the millimolar range, the relatively high concentration of PDI required to achieve a high yield of reactivation is not surprising. The time period needed for maximum reoxidation (approx. 3 days) is perhaps indicative of the method of action of PDI in this manner *in vitro*, which appears to be the reshuffling of incorrectly formed disulphide bridges into the correct native links. The rate limiting step in this process could reflect the rate at which non-native links become accessible to PDI (McClelland *et al.*, 1995). In the case of RfBP, with 9 disulphide bonds, it is extremely unlikely that every incorrectly formed such bond is accessible to the action of PDI at any one time. The slow rate of reoxidation could reflect (i) the need for the protein to unfold, at least partially, in order to allow access to the PDI, and/or (ii) the very large number of combinations of incorrectly formed disulphide bonds which PDI would have to shuffle, in order to reach the native form - 18 free sulphide groups can theoretically form over 34 million disulphides. Although in practice this number will be reduced due to steric and spatial considerations, the number of possible combinations will still be vast.

Even the presence of PDI from the very start of the reoxidising process does not facilitate the formation of the correct disulphides in anything approaching the physiological time scale, i.e milliseconds-seconds. Therefore, while PDI is undoubtedly a useful tool *in vitro* for enhancing yields in the reoxidation of reduced proteins by the 'shuffling' of incorrectly formed disulphides, it is clear that its role *in vivo* can only be understood in the context of the other processes and factors which are involved in the

production of correctly folded proteins. It is thought that *in vivo* at least some of the disulphide bonds are formed co-translationally, and that some initial folding/secondary structure formation occurs within the nascent polypeptide chain (Bergman and Kuehl, 1979; Peters and Davidson, 1982). It is possible that various chaperone proteins found in the lumen of the ER interact with the folding protein chain and these could bring pairs of sulphhydryl groups into juxtaposition so that the correct disulphides are formed in a co-translational manner by the action of PDI. It has been suggested that in RfBP, the breaking of only one (unidentified) disulphide bond can lead to the loss of the binding activity of the protein (Kozik, 1982a). In the reverse process correct formation of this single bond could possibly have a crucial effect on the subsequent formation of the correct disulphide bonds within the molecule. The action of PDI in this putative role *in vivo* would obviously be much quicker than its isomerisation function in this type of experiment.

It can be seen therefore that the conditions under which the *in vitro* reoxidation process involving the completed polypeptide chain is studied are different from those which pertain to the biosynthesis of the protein. *In vivo*, disulphide bond formation may occur as the polypeptide chain is growing and forming localised structural elements, and additional components such as chaperone proteins are present. However, given that the oxidation of reduced RfBP to give reasonable yields of active protein can be achieved in the presence of PDI, albeit on a greatly retarded time scale, it is reasonable to suggest that studies such as this can give important insights into the interplay between protein folding and disulphide bond formation in secreted proteins. This is particularly true for those proteins (such as RfBP) where reoxidation of the reduced proteins using mixtures

of GSH and GSSG alone gives very poor yields of active material.

Chapter 6 Dephosphorylation of RfBP

6.1 Introduction

Phosphorylation is the most common reversible covalent post-translational modification undergone by proteins (Darby and Creighton, 1993). It involves the substitution of an amino acid side chain by a phosphate group. Serine, threonine and tyrosine are the most commonly phosphorylated residues, but aspartic acid, histidine and lysine can also be modified. The reaction can be catalysed by residue specific and non-specific kinases (Creighton, 1993).

The reversible phosphorylation of proteins and enzymes by kinases and phosphatases plays a number of important roles including cellular control, regulation and transport (Stryer, 1988). The phosphorylation or dephosphorylation of enzymes can act as a switch, turning on the activity of that enzyme, which in turn can similarly modify another molecule, often resulting in a cascade of activation. For example, skeletal muscle phosphorylase exists in two interconvertible forms, *a* and *b*. The dimeric, inactive phosphorylase *b* is converted to active phosphorylase *a* upon the phosphorylation of serine 14 in each subunit (Stryer, 1988). This reaction is catalysed by phosphorylase kinase, itself regulated by phosphorylation (Stryer, 1988). Phosphorylation can also act as a marker for protein targeting. The formation of a mannose 6-phosphate residue by a phosphotransferase is vital for the targeting of glycoproteins from the Golgi to the lysosome (Stryer, 1988).

6.1.2 Phosphorylation of apo-RfBP

Egg white apo-RfBP contains 8 phosphorylated serine residues (Rhodes *et al.*, 1959; Miller *et al.*, 1982). These are situated in a very anionic region between residues 186 and 199 that also contains five glutamates and one methionine residue (Fig. 1.1) (Hamazume *et al.*, 1984).

Dephosphorylation of RfBP was first reported by the group who first isolated and purified RfBP from hen egg white (Rhodes *et al.*, 1959). Potato acid phosphatase was used, and 95% dephosphorylation was reported. It was found that dephosphorylation had no effect on the binding of riboflavin by the protein. More recently, *in vivo* studies involving the partial dephosphorylation of yolk RfBP have demonstrated that the removal of just one phosphate decreases uptake from the serum to the yolk (by 60 %), and subsequent dephosphorylation further decreases uptake (Miller *et al.*, 1982).

Studies have shown that the native protein can bind calcium phosphate and form cross links with other RfBP molecules, but this property is greatly impaired on dephosphorylation (Aoki *et al.*, 1993). In the past, the search for a specific receptor for serum RfBP, to facilitate uptake into the yolk proved fruitless (White and Merrill, 1988). However, recent studies have shown that RfBP can form a complex with vitellogenin in the presence of calcium phosphate (MacLachlan *et al.*, 1994). It is thought that RfBP utilises the uptake of vitellogenin by the 95 kDa lipoprotein receptor, in a in a sort of "piggy-back" transport system, to gain entry to the yolk (Fig. 6.1).

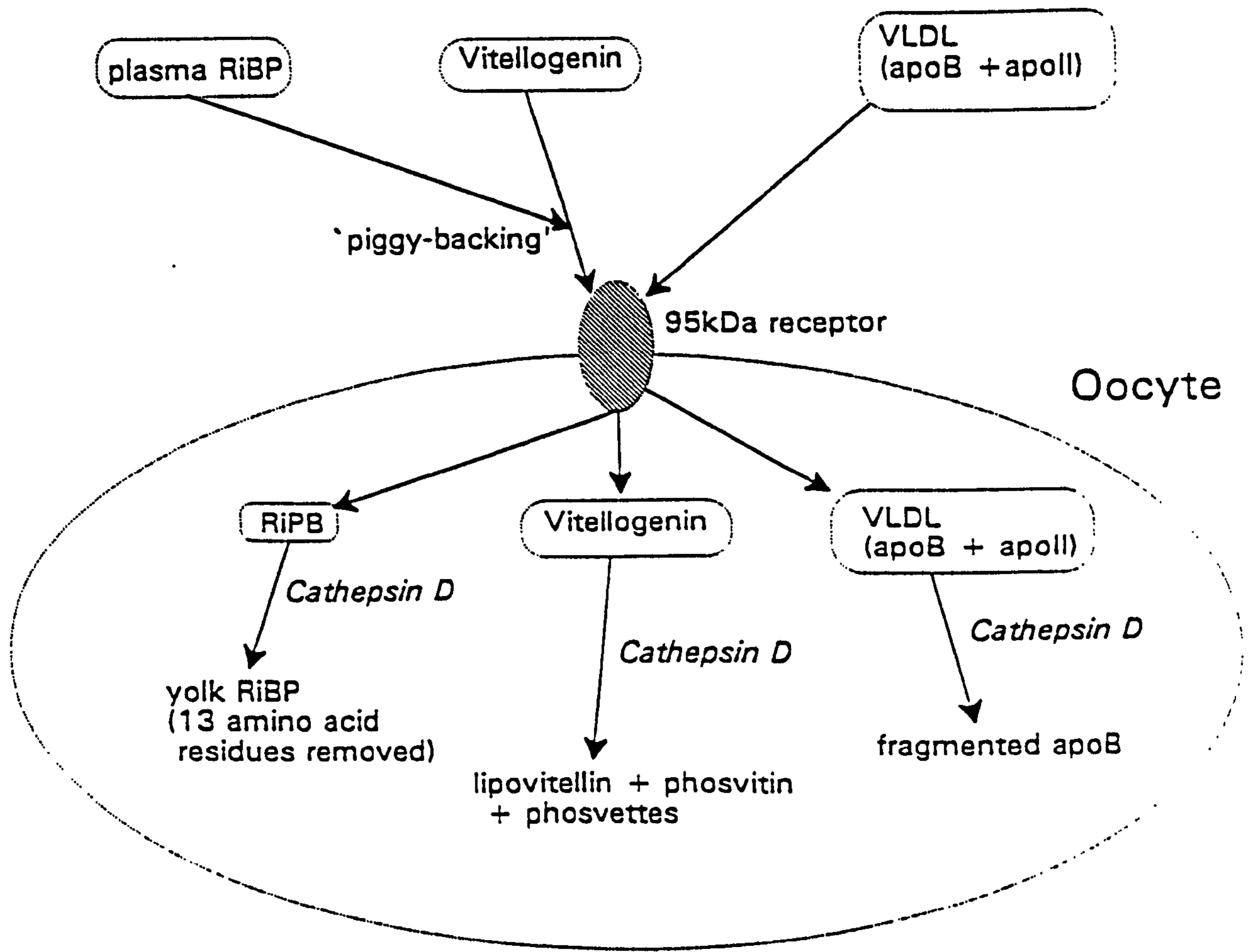


Fig 6.1 The roles of the 95kDa receptor protein and cathepsin D in the uptake and modification of RfBP, and other yolk proteins (from Stevens, 1996)

6.2 Methods

6.2.1 Dephosphorylation of RfBP

The method used was that of Rhodes *et al.* (1959). 1.5 ml of a 2 mg/ml RfBP solution was dialysed against 0.05 M sodium acetate, pH 5.3. The dialysis tubing was then placed in a universal tube filled with 30 ml of buffer, at 37°C. 15 µl of potato acid phosphatase (0.9 units) (Boehringer Mannheim, grade 1) was added to the RfBP solution in the dialysis tubing, followed by an additional 5 µl (0.3 units) after 3 hours incubation. 1 ml of buffer (outside the dialysis bag) was removed from the universal tube and retained for inorganic phosphate determination. Further 1 ml samples were taken from the buffer at 1 hour intervals for the next 4 hours, and a final sample the following morning.

The RfBP/phosphatase solution was removed from the dialysis sac and loaded onto a Whatman DE52 ion exchange column (1 x 8 cm, preswollen in buffer). The column was washed with 0.05 M sodium acetate buffer, pH 7, until the A_{280} of the effluent was equal to that of the buffer (the acid phosphatase is not retained by the column). The bound RfBP was eluted with buffer containing 0.5 M NaCl. For studies of refolding, the sample was freeze-dried and resuspended to a concentration of 10 mg/ml.

6.2.2 Inorganic phosphate determination

The samples taken from the buffer in the universal tube were tested for inorganic phosphate (released from RfBP by the acid phosphatase enzyme) (adapted from Ames, 1959). 0.5 ml 2% ammonium molybdate and 0.5 ml 10% ascorbic acid (both in H₂O) were added to the 1 ml samples. A buffer blank was also prepared. The samples were

mixed well, boiled for 5 minutes, cooled (30 min) and the absorbance at 800nm recorded. A standard phosphate curve was produced, using 2.5 mM - 62.5 mM KH_2PO_4 . The standards were treated in the same manner as the samples.

6.2.3 Phosphate stain for SDS gels

15 μl of dephosphorylated and native RfBP were subjected to SDS polyacrylamide gel electrophoresis, as previously described (Section 2.4). The gel was stained for the presence of phosphates, as described by Cutting (1984). The following reagents were prepared:

- A. 10% (w/v) sulphosalicylic acid in 25% 2-propanol
- B. 0.5M CaCl_2 in 10 % sulphosalicylic acid
- C. 0.5M NaOH
- D. 1% (w/v) ammonium molybdate in deionised water
- E. 1% (w/v) ammonium molybdate in 1 N HNO_3
- F. 0.5% methyl green in 7% acetic acid
- G. 10% (w/v) sulphosalicylic acid
- H. 7% acetic acid

SDS gels were fixed in A for 12hr, or overnight, with frequent changes of fixer. The gel was fixed in B for 1 hr, and then rinsed thoroughly in H_2O (to reduce background). The gel was then placed in C, preheated to 60°C, for 30 min, then rinsed 2 x 10 min

in reagent D. The gel was then placed in E for 30 min, then stained in F for 30 min. The gel was destained in G, at 60°C. The destained gel was stored in H.

6.2.4 Characterisation and refolding of dephosphorylated RfBP

The dephosphorylated protein was run on an SDS polyacrylamide gel alongside native protein, and stained for protein. CD and fluorescence spectra of the dephosphorylated RfBP were recorded (Sections 2.2.1 and 2.3.1). The riboflavin binding ability of the dephosphorylated protein was ascertained (Section 2.8).

6.2.4.1 Refolding of denatured dephosphorylated RfBP

The dephosphorylated RfBP was denatured as before (Section 4.2.1). The refolding process was studied in the presence and absence of riboflavin, using both steady state and stopped-flow instrumentation.

6.2.5 ANS as a probe for structural changes in dephosphorylated RfBP

The interaction between denatured, refolding and folded dephosphorylated RfBP was studied, as described in Section 4.2.3.

6.3 Results

6.3.1 Dephosphorylation of RfBP

On average, after dephosphorylation and separation from the phosphatase enzyme, the process yielded approximately 55 ± 5 % RfBP.

The values obtained for the phosphate standard curve can be seen in Table 6.1, and in

Figure 6.2. The inorganic phosphate determinations on the samples of buffer removed during the phosphatase incubations can be seen in Table 6.2 and Figure 6.3.

6.3.2 SDS Gel analysis

The native protein was tested positive for the phosphate stain, while the dephosphorylated protein did not stain. When both versions of the protein were stained with Coomassie reagent, the dephosphorylated protein, had ran slightly further on the gel, corresponding to a loss of mass.

6.3.3 Spectroscopic analysis

The CD spectrum of dephosphorylated RfBP was slightly different from the native spectrum (Fig. 6.4). CONTIN procedure analysis (Provencher and Glöckner, 1981) revealed a reduction in the α -helical content. The fluorescence spectrum for dephosphorylated RfBP was virtually identical to the native spectrum (Fig. 6.5). The dephosphorylated enzyme bound riboflavin in a 1:1 ratio, in an identical fashion to the native protein (Fig. 6.6). The dephosphorylated protein appeared to unfold in a similar manner to the native protein (Figs. 6.7, 6.8).

6.3.4 Refolding of dephosphorylated RfBP

6.3.4.1 Protein fluorescence and riboflavin binding

Using manual mixing with the Perkin Elmer LS 50 instrument, the pattern of refolding was identical to that of the native, with a very long slow phase taking over an hour to completely regain protein fluorescence (Fig. 6.9).

Table 6.1 Absorbance values for phosphate standards.

Phosphate (nmole)	A_{800}
2.5	0.004
12.5	0.070
25.0	0.173
37.5	0.266
50.0	0.366
62.5	0.469

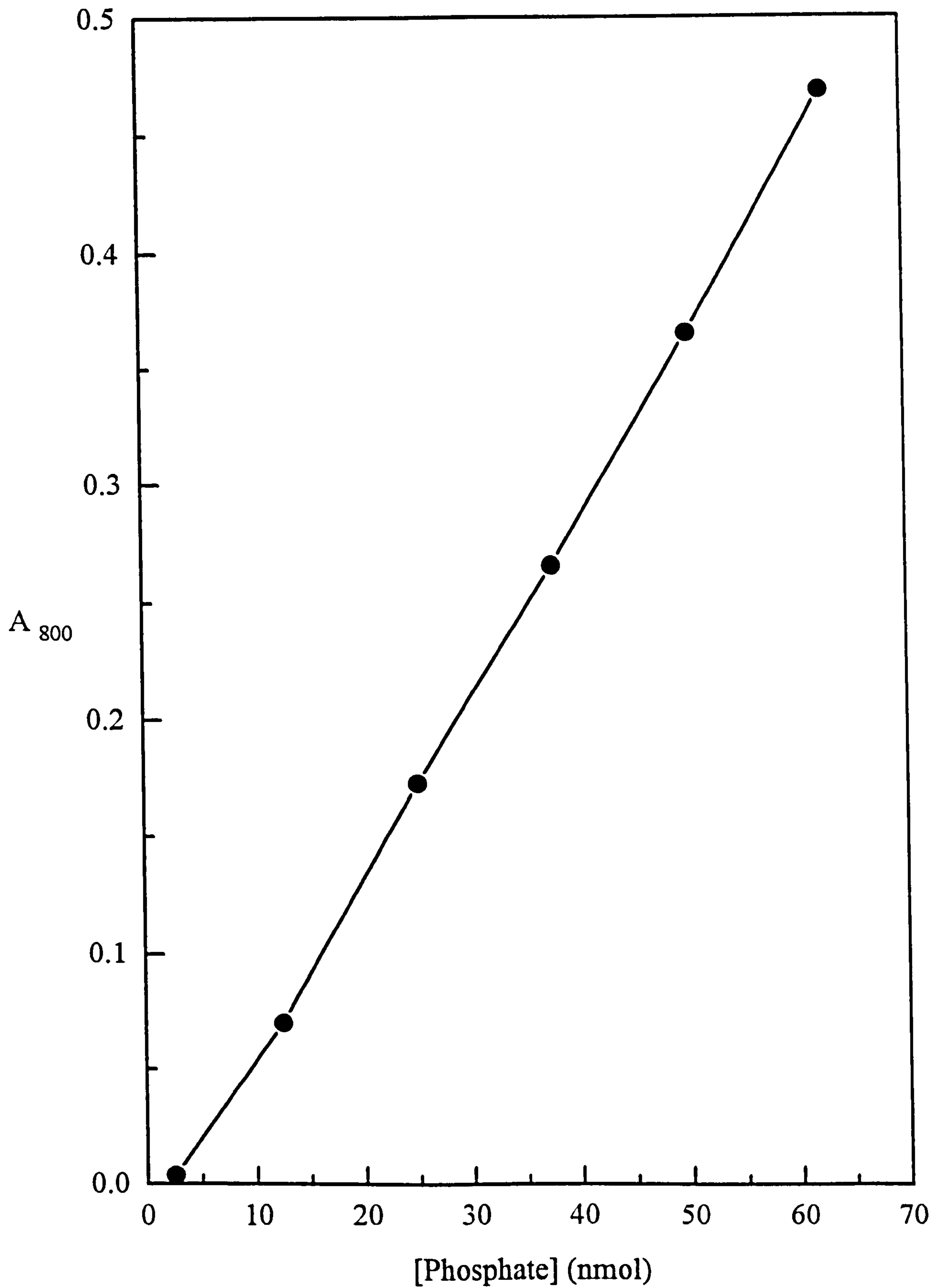


Figure 6.2 Standard curve for free phosphate estimation

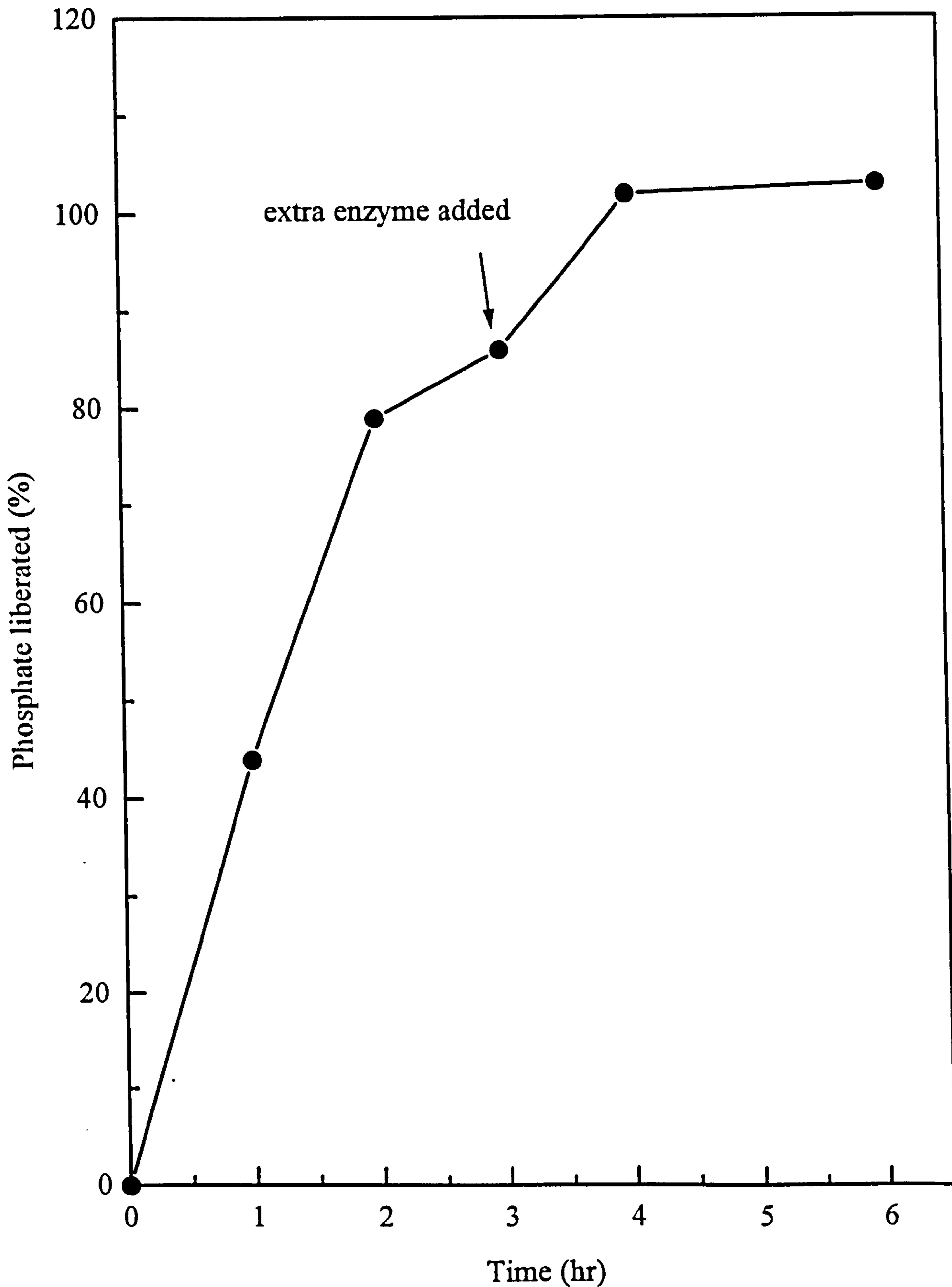


Figure 6.3 Enzymatic release of phosphates from apo-RfBP using potato acid phosphatase

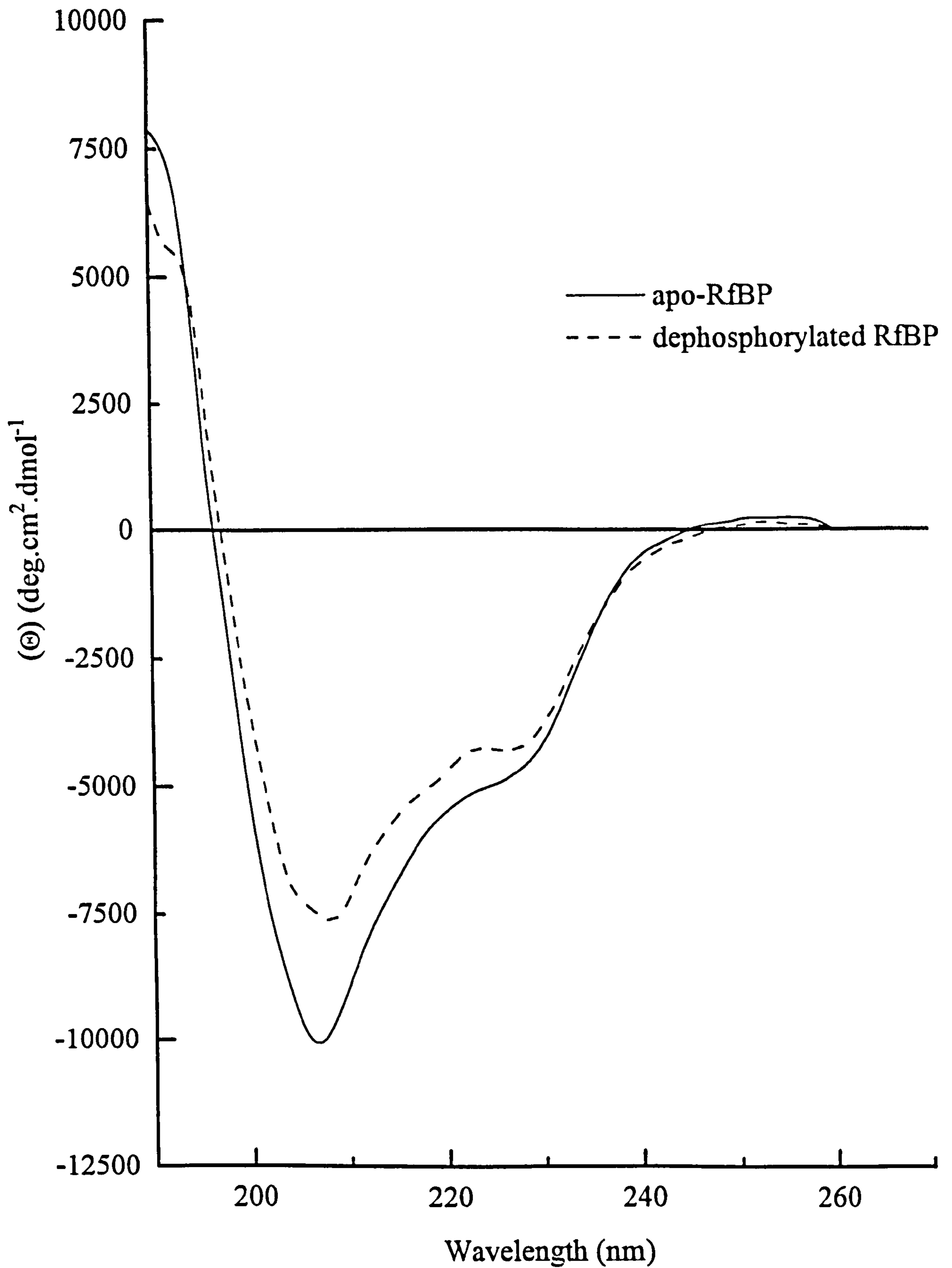


Figure 6.4 The far UV CD spectra of native and dephosphorylated RfBP

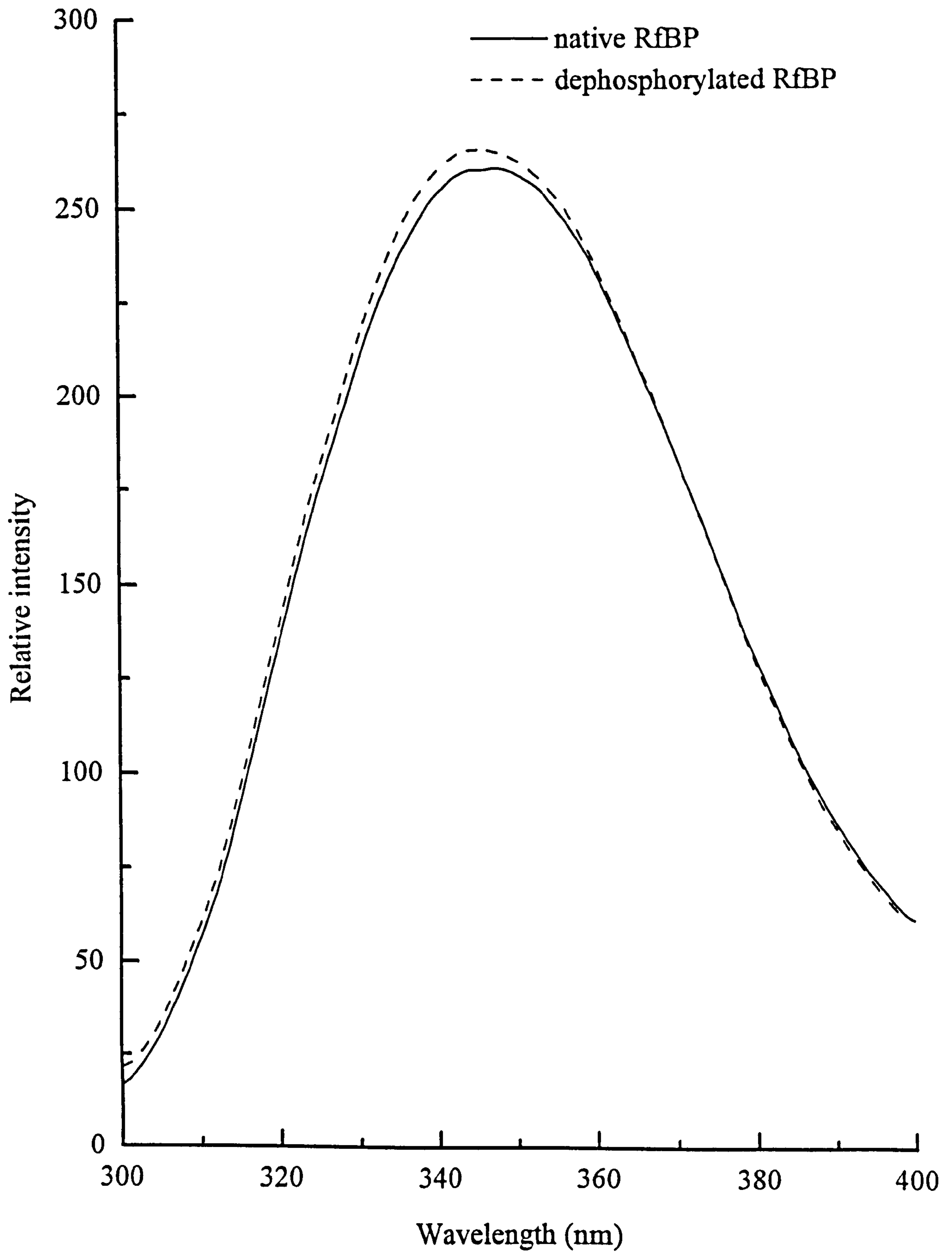


Figure 6.5 The fluorescence spectra of native and dephosphorylated RfBP

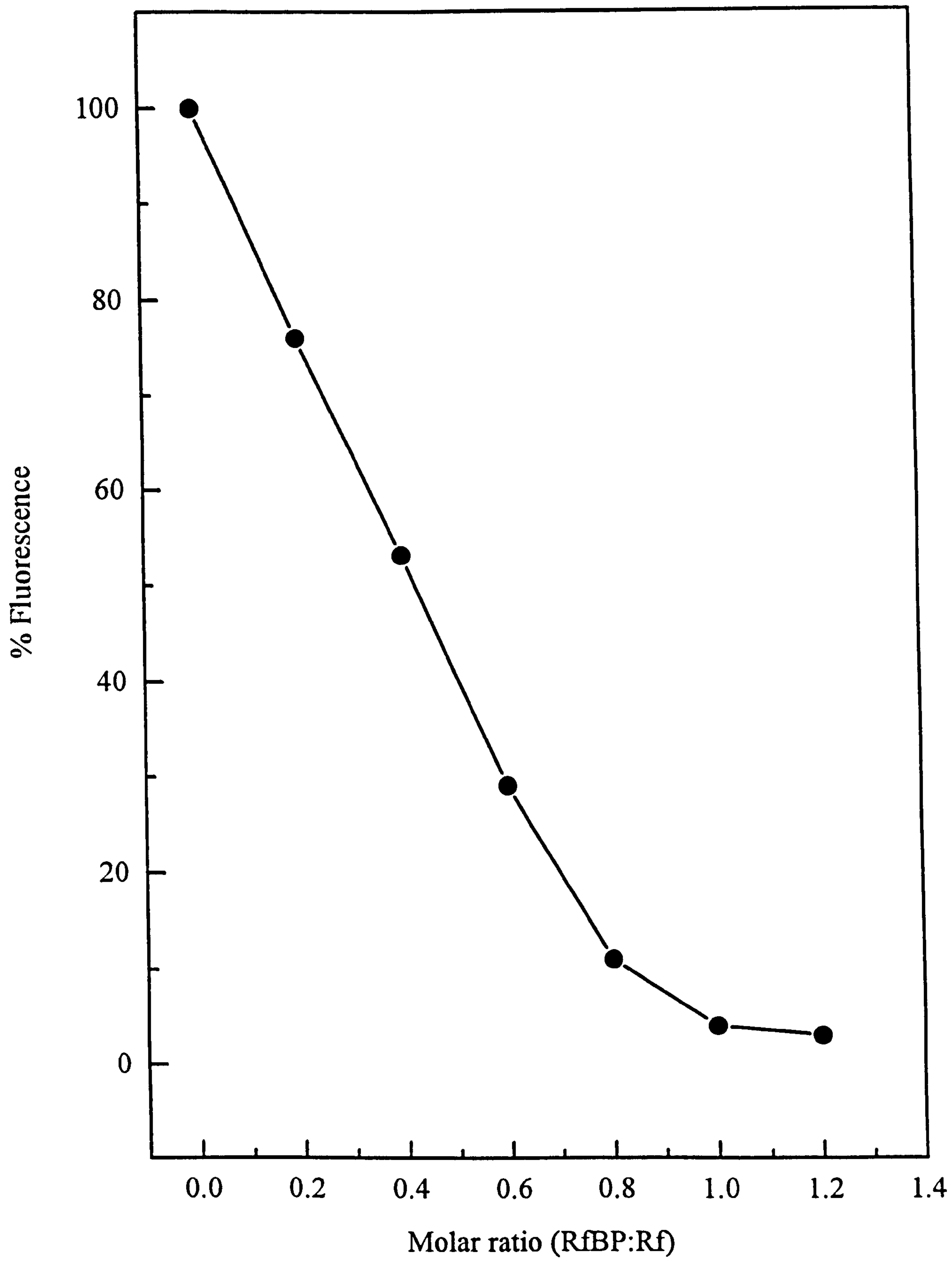


Fig 6.6: The quenching of riboflavin fluorescence upon binding to dephosphorylated RfBP.

Figure 6.7 Changes in the CD spectrum of dephosphorylated RfBP after incubation in 0-6 M Gdn HCl

2.000E+00

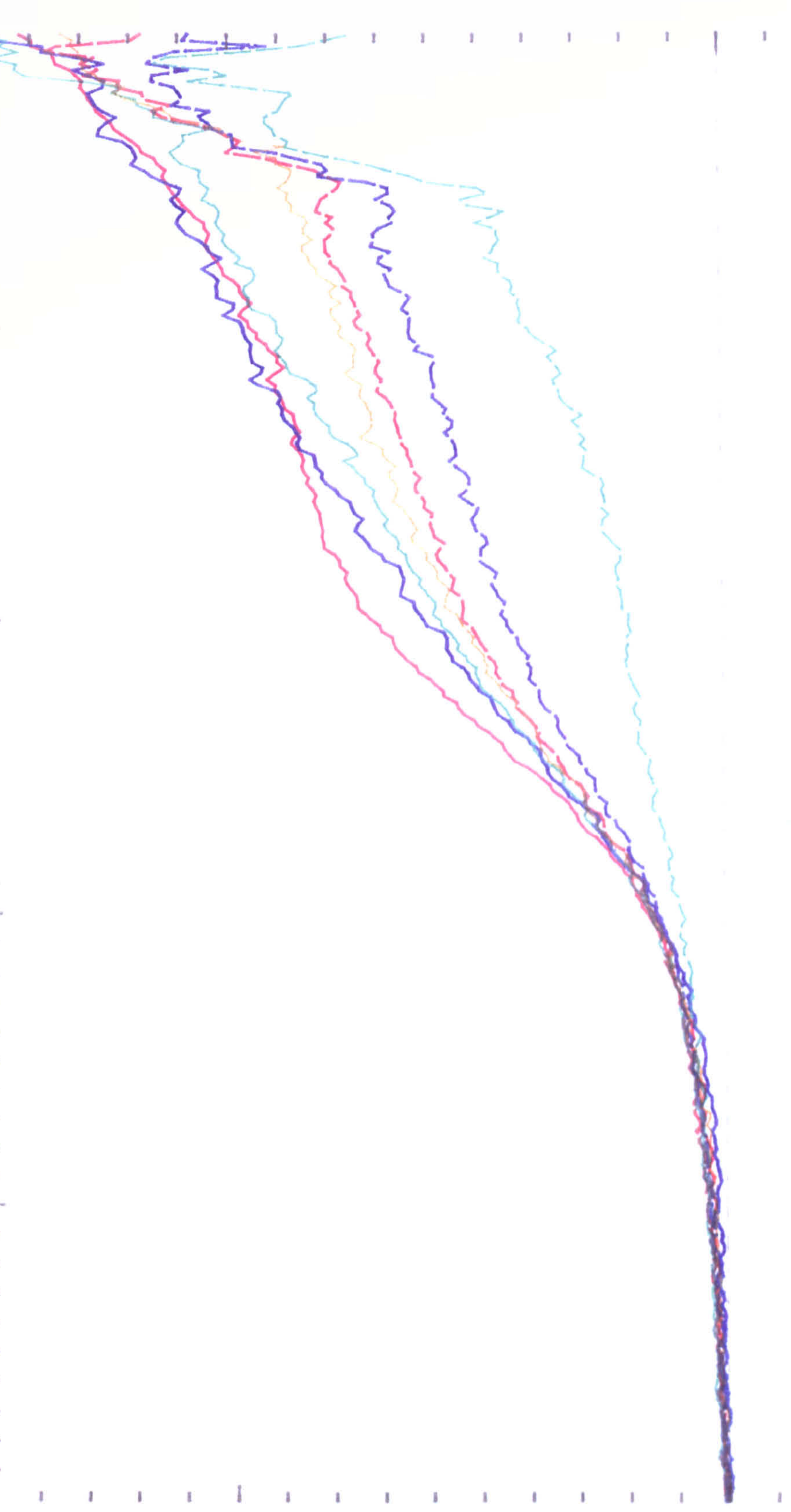
CD
[mdeg]

-1.500E+01
210.0

WL [nm]

260.0

- 1: DE-PHOS AFBP 0MGDN
- 2: DE-PHOS AFBP 1MGDN
- 3: DE-PHOS AFBP 2MGDN
- 4: DE-PHOS AFBP 3MGDN
- 5: DE-PHOS AFBP 4MGDN
- 6: DE-PHOS AFBP 5MGDN
- 7: DE-PHOS AFBP 6MGDN



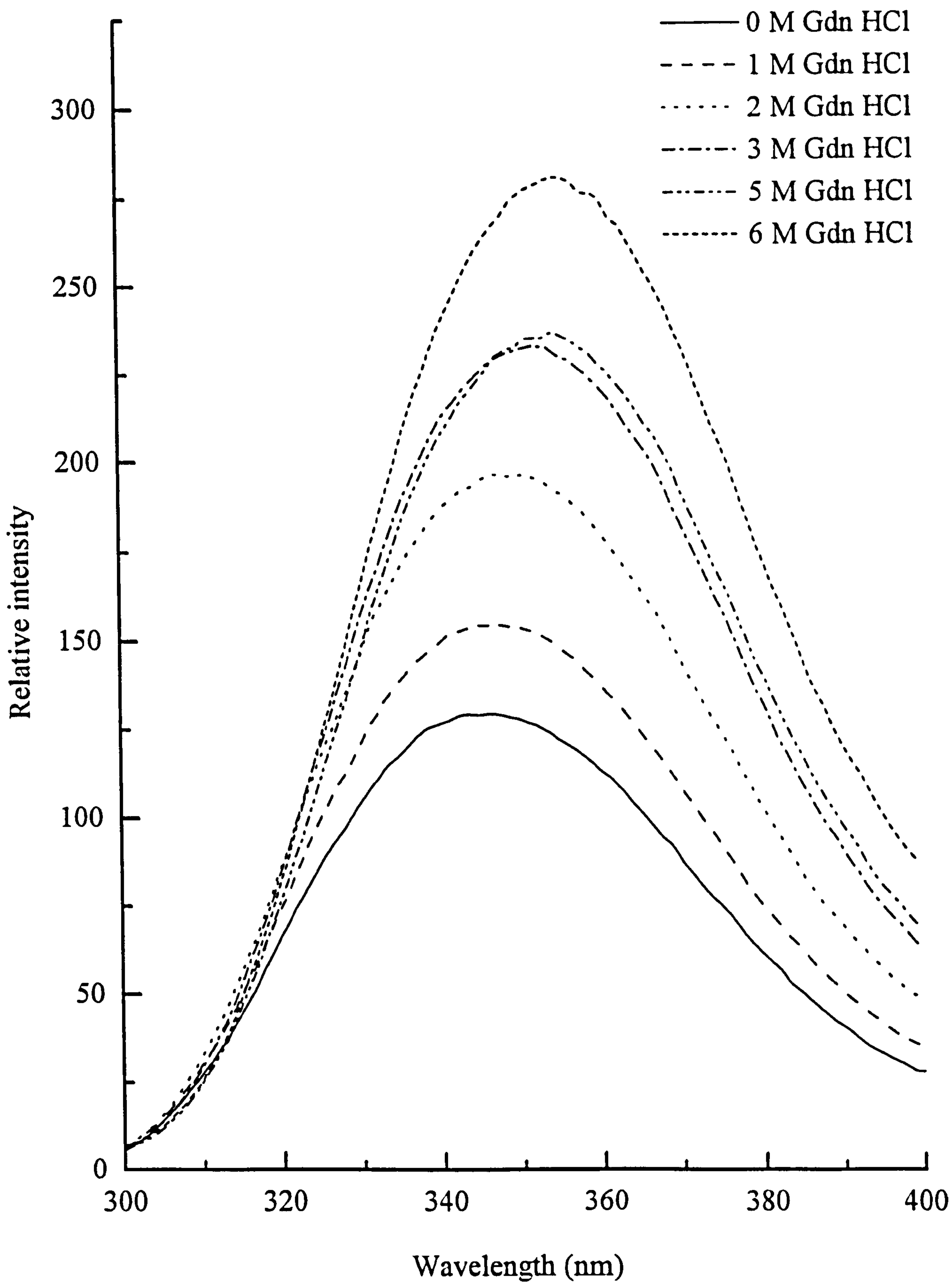


Figure 6.8 Changes in the fluorescence spectrum of dephosphorylated RfBP after incubation in 0-6 M Gdn HCl

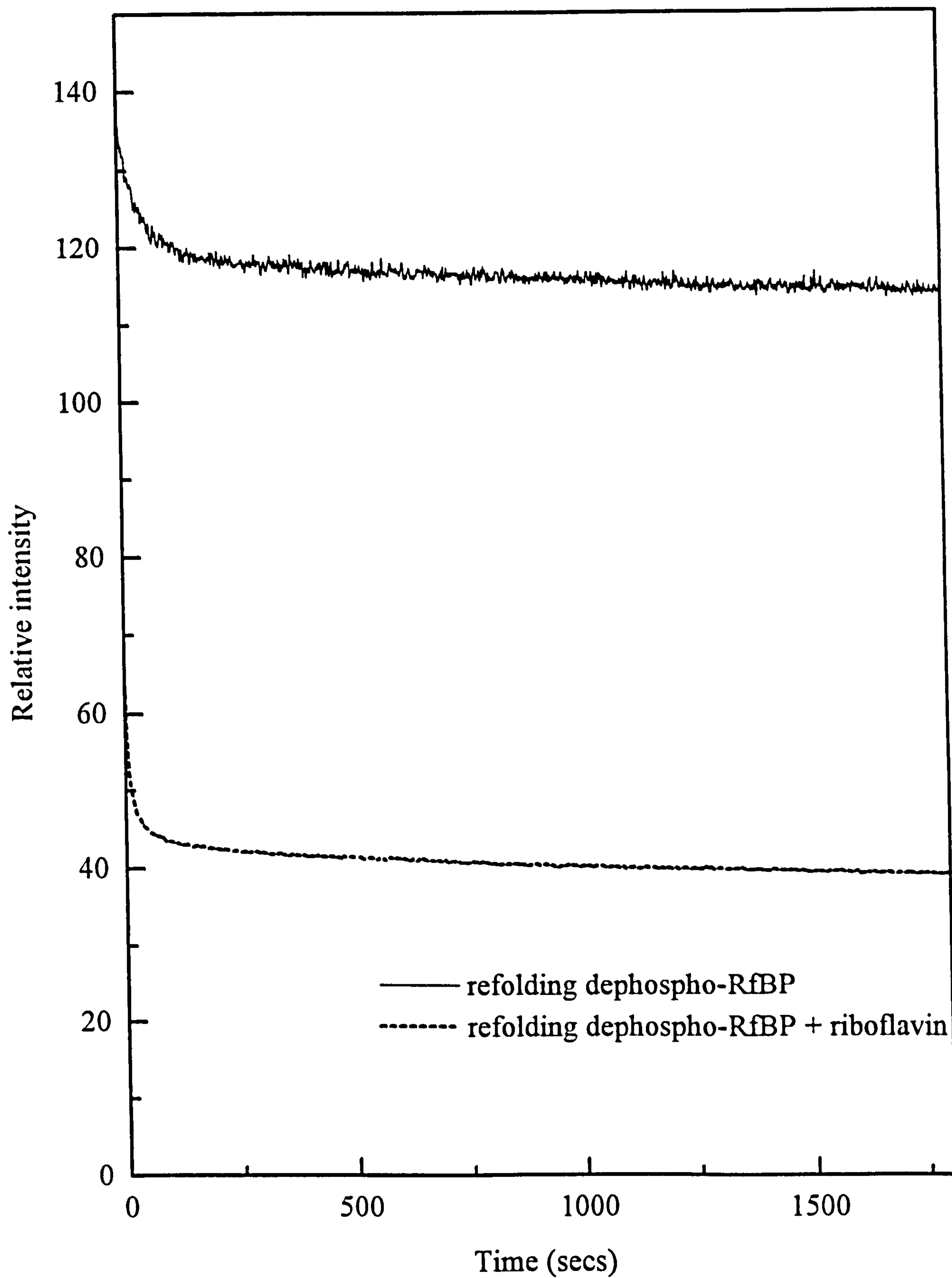


Figure 6.9 Fluorescence emission at 350nm following dilution of Gdn HCl, in the presence and absence of riboflavin

The analysis of the refolding reaction of the dephosphorylated apo-protein, as measured by the stopped-flow instrument, indicated that it followed similar kinetics to those of the native apo-protein.

6.3.5 ANS binding

The pattern of ANS binding followed a similar pattern to that of native RfBP, as described in 4.3.3. It was noted, however, that the enhancement of ANS fluorescence upon binding the dephosphorylated protein was approximately 10% greater than the enhancement found with the native protein (Fig. 6.10).

6.4 Discussion

RfBP was readily dephosphorylated by the potato acid phosphatase, the inorganic phosphate analysis revealing that all eight phosphoserines were hydrolysed. The fluorescence spectrum was identical to that of the native protein, and the riboflavin binding ability is not affected by dephosphorylation. However the secondary structure, as estimated from the CD spectrum by applying the CONTIN procedure (Provencher and Glöckner, 1981) showed a marked decrease in α -helix content. It is possible that the loss of these eight negatively charged groups, clustered together, leads to a change in local ionic interactions, causing a slight relaxation in the local secondary structure. From the fluorescence and binding analysis, however, it is clear that no gross overall structural change occurs upon dephosphorylation, and that the binding site is conserved.

The kinetics of the refolding reaction for dephosphorylated RfBP, in the presence and absence of riboflavin, were found to be identical to that of the native protein. Likewise,

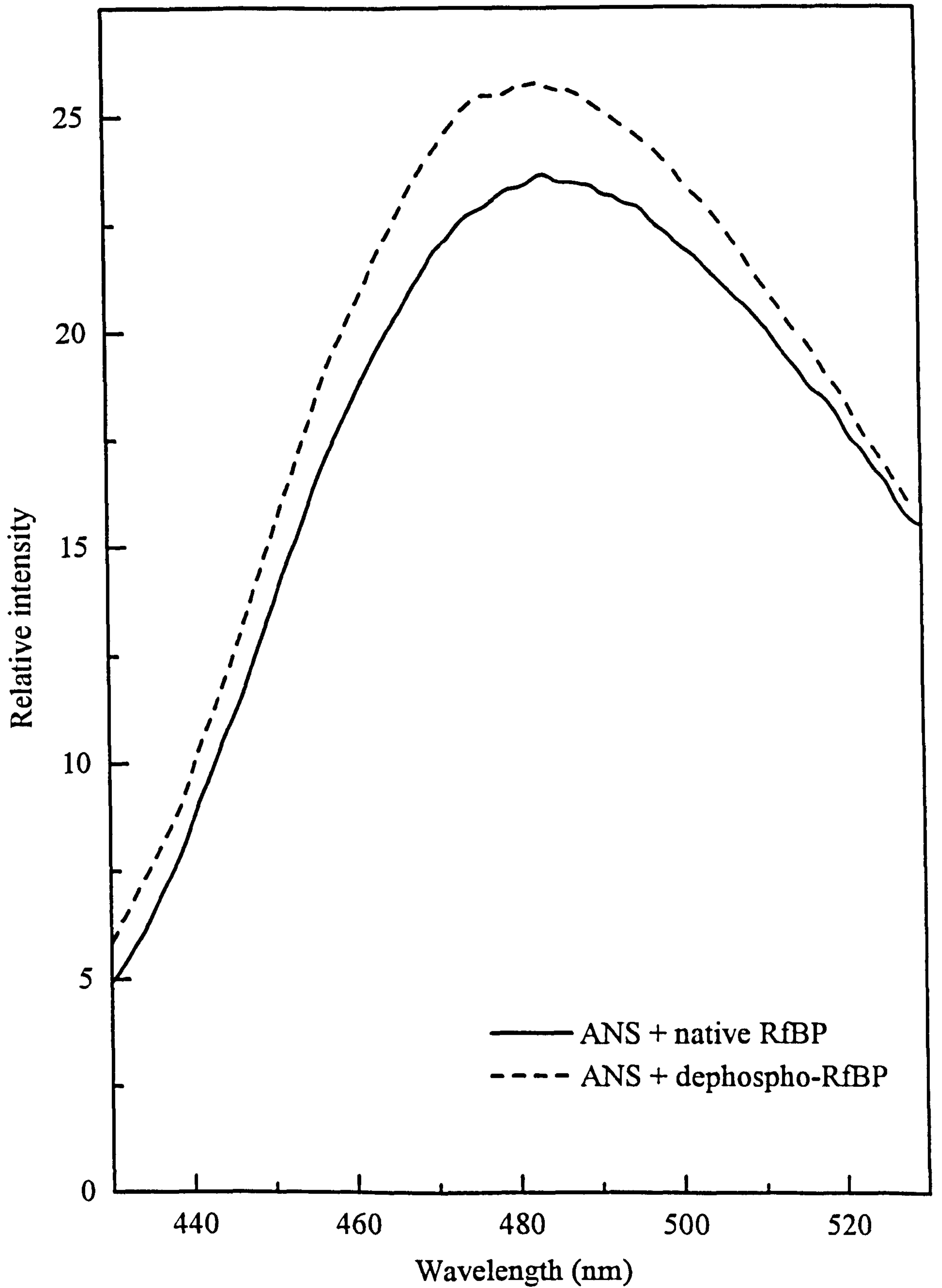


Figure 6.10 The fluorescence spectra of ANS in the presence of native and dephospho-RfBP

the kinetics for the regain of riboflavin binding activity by refolding and the native protein. It was thought possible that the cleavage of the phosphate groups may have had some effect on the refolding of RfBP *in vitro*, as with the loss of 8 negatively charge groups, the local ionic interactions could have been disrupted, or enhanced. Electrostatic attractions or repulsions could possibly reduce the vast number of conformations open to the random coil unfolded molecule. However, dephosphorylation had no effect on the refolding of RfBP. Taken together with the accessibility of the phosphate groups to the phosphatase enzyme, this suggests that the phosphates are located on the exterior of the protein, and that the phosphorylation occurs after folding has completed.

It seems likely that the phosphates are involved in cross-linking the protein to vitellogenin, for "piggy back" transport into the yolk via the 95 kDa receptor (MacLachlan *et al.*, 1994). All three forms of RfBP (white, yolk and serum) are able to bind equally well to vitellogenin (MacLachlan, *et al.*, 1994). However, the rate of clearance of the white and yolk RfBPs from the blood (as opposed to uptake by the developing oocyte) in tracer studies is much greater than for the serum protein (Miller *et al.*, 1982a). This is probably due to the difference in carbohydrate structures, targeting the yolk and white for preferential uptake by the liver, from the bloodstream, for degradation (Miller *et al.*, 1982)(Section 7.1.2).

Chapter 7 Deglycosylation of RfBP

7.1 Introduction

Glycosylation is the most common and diverse post-translational modification undergone by proteins (Lis and Sharon, 1993). From higher organisms, to archaebacteria, the process is virtually ubiquitous. Almost all egg white proteins and blood serum proteins are glycosylated for example (Lis and Sharon, 1993). Glycoproteins can be found in the cytoplasm, the nucleus or be membrane-spanning (without exception, integral membrane proteins of higher organisms are glycosylated). Some bacteria, long thought to be unable to glycosylate proteins, have now been shown to possess the ability to form glycoproteins (Lis and Sharon, 1993). Although there is a relatively small number (around 10) of monosaccharides available, the structural variety possible is huge, with different linkages (i.e. α or β), anomeric stereochemistry, branching etc.

Due to the fact that the carbohydrate structure is not dictated by the gene sequence, glycosylation can be tissue, or even cell, specific, leading to functional diversity from a single gene product. For example, HLA-DR antigen isolated from a human melanoma cell line and from a B-lymphoblastoid cell line, both originating from the same individual was found to be identical, except for the size of the carbohydrates and the extent of sialylation (Alexander *et al.*, 1984). Also, the glycosylation of Asn 245 of viral surface glycoprotein E1 of Sindbis virus depends on the viral host- whether the virus is grown in chick embryo fibroblasts or Chinese hamster ovary cells - interestingly, glycosylation of the other site (Asn 139) is unaffected by the cell-type (Hsieh *et al.*, 1983). Tissue and cell specific glycosylation such as this has implications

for antibody recognition.

The significance and function of glycosylation are only just beginning to be understood. Carbohydrates are thought to be involved with the removal of glycoproteins from the blood- the terminal sialic acid residue is removed by sialylases in the blood stream after a passage of time (Lis and Sharon, 1993). The exposed galactose residue is then bound to receptors on the membranes of liver cells, which causes them to be internalised (endocytosis) for degradation. Carbohydrates have also been implicated in cell-cell interactions, *i.e.* interactions between neural cell adhesion molecules (N-CAM) (Lis and Sharon, 1993). Glycosylation may also play a role in the stabilisation of protein structure, increasing their life span. Yeast invertase is produced as a number of isoenzymes, differing only in the extent of glycosylation (a greater degree of glycosylation, in part greater resistance to degradation (Candy, D.J, 1980)). Also, over 50% of monoclonal antibodies generated against animal cells and cell membrane proteins have been shown to recognise carbohydrates of surface proteins (Lis and Sharon, 1993).

7.1.2 Glycosylation of apo-RfBP

Hen egg white RfBP contains two complex carbohydrates N-linked to asparagines 36 and 147 (Hamazume *et al.*, 1984)(Fig. 1.1). Although the carbohydrate composition is known, the structure has not yet been determined (Miller *et al.*, 1982a). The main structural difference between the white, yolk and serum riboflavin binding proteins is found in the carbohydrate composition. Although the serum protein becomes the yolk protein upon uptake by the oocyte, significant differences in carbohydrate composition

have been found between them, suggesting that serum RfBP must undergo glycolytic modification during or after transport to the yolk (Miller *et al.*, 1982a). The differences in carbohydrate composition were thought to be due to a carbohydrate-directed uptake mechanism, for the transport of serum RfBP to the yolk (Miller *et al.*, 1981). However tracer studies have shown this not to be the case (Miller *et al.*, 1982a) When labelled serum, yolk and white RfBPs were injected into hens, it was found that the low proportions of white and yolk RfBP reaching the oocyte reflected preferential clearance from the plasma, rather than specific uptake of the serum protein. It seems likely that this is due to a hepatic receptor which recognises the terminal N-acetylglucosamine of the white (Miller *et al.*, 1982a). The phosphates are currently thought to be involved in the uptake of serum RfBP into the yolk (discussed further in Section 6.4).

7.1.3 Deglycosylation of RfBP

A chemical method of deglycosylation was chosen for RfBP. The method of Edge *et al.* (1981) was adopted over the hydrogen fluoride (HF) technique, largely for safety considerations. This method involves the use of trifluoromethanesulphonic acid at low temperature, followed by the neutralisation of the acid by aqueous pyridine, which has been found to be efficient in the cleavage of N-linked carbohydrates from proteins, without detriment to the native protein structure (Sojar and Bahl, 1987).

This method has previously been applied to RfBP by Karande *et al.*, (1991) who reported successful deglycosylation (as shown by lack of staining for carbohydrate on SDS PAGE gels), although they reported some possible denaturation of the protein. The neutralisation reaction between TFMS and pyridine is extremely exothermic, and

therefore, in the modified method employed, every effort was used to keep the reaction temperature at a minimum. The lack of staining for the presence of carbohydrates on gels was used as an indicator of successful deglycosylation.

7.2 Methods

7.2.1 Chemical deglycosylation of apo-RfBP

The chemical deglycosylation method employed was modified from Sojar and Bahl (1987). 60 µl of toluene was added to 1 ml of trifluoromethanesulphonic acid (TFMS) and mixed gently. 1 mg samples of RfBP (lyophilised in a glass test tube) was placed on an ethanol/dry ice for 30 seconds. 150 µl of TFMS/toluene solution was added very slowly to the protein, and the reaction mixture left on the ethanol/dry ice for 30 seconds, then transferred to a -20°C freezer for 10 mins. The samples were removed and shaken gently to aid melting. They were replaced in the -20°C freezer, and left for up to 4 hours. The samples were then placed on ethanol/dry ice for 30 seconds. 450 µl of 60% pyridine was added very slowly, and left to stand for 30 seconds, then transferred to dry ice for 5 minutes, then to an ice/water bath for 15 minutes. 1.5 ml of 0.5% ammonium bicarbonate was added to the test tube, and mixed. The solution was transferred to an Eppendorf tube, and the precipitate formed on ammonium bicarbonate addition was pelleted by centrifugation (15 mins). The supernatant was discarded, the pellet resuspended in 1 ml 0.5% ammonium bicarbonate, and dialysed against this solution, to remove the pyridine. The sample was then dialysed overnight against 0.05 M sodium phosphate buffer, pH 7.0.

7.2.2 SDS PAGE analysis of deglycosylated apo-RfBP

The deglycosylated samples were ran on an SDS PAGE gel (Section 2.4), along with native RfBP. The gel was then stained for the presence of carbohydrate and protein (Coomassie Blue and silver staining) using the following methods:

Carbohydrate Staining

The carbohydrate stain procedure was that of Sargent and George (1975).

Reagents: 12.5 % TCA
 Schiff's reagent
 0.5 % sodium metabisulphite
 5 % acetic acid
 1 % periodic acid/3 % acetic acid

The gel was immersed in the TCA for 30 minutes, then rinsed in distilled H₂O for 30 minutes. It was then washed with the periodic/acetic acid solution for 50 minutes, then water overnight. The gel was stained by immersion in Schiff's reagent for 50 minutes in a dark room, and then washed with sodium metabisulphite (3 x 30 min) to remove excess stain, followed by an overnight immersion in water. The gel was stored in 5% acetic acid.

Silver Stain

The silver stain procedure was adapted from that of Gottlieb and Chavko (1987). The method was as follows:

Reagents:

- Fixer - 50 ml methanol
- 10 ml acetic acid
- 10 ml 50 % v/v glycerol
- 30 ml distilled H₂O

Stain and development- A. 2 % silver nitrate/2 % ammonium nitrate

B. 10 % tungstosilicic acid

C. 2.8 % v/v formaldehyde (n.b. commercial stock is 37 %)

D. 5 % sodium carbonate (store at 4°C, use within 3 months)

A, B and C are made w/w with distilled H₂O

The gel was shaken gently in fixer for 20 minutes, and then in 200 ml of distilled H₂O twice for 10 min. The staining solution was prepared thus:- 17.5 ml distilled H₂O was placed in a beaker. 2.5 ml of each of A, B and C, were added in that order, while mixing rapidly. Then 25 ml of D was added to the beaker quickly, still mixing rapidly.

The water was then decanted from the gel and the stain solution added. The gel was shaken gently until bands appeared (5-15 min). The staining reaction was stopped by placing the gel in 5 % acetic acid. The gel was stored in the 5 % acetic acid.

7.2.3 Characterisation of deglycosylated apo-RfBP

The sample was subjected to CD and fluorescence analysis, as described previously (Sections 2.2.1 and 2.3.1). A riboflavin binding assay was also carried out on the deglycosylated sample (Section 2.8).

7.3 Results

7.3.1 SDS PAGE analysis of deglycosylated apo-RfBP

Initially it appeared as if the deglycosylation of RfBP had been successful- upon staining of one half of the gel for carbohydrate, the native controls tested positive for the presence of sugars, while the lanes containing the deglycosylated sample were clear of staining. However, when the other half of the gel was stained for protein, the deglycosylated sample lanes did not stain. The absorbance of the deglycosylated sample at 280 nm indicated that there was protein present (although the absorbance at 310 nm was relatively high (approx. 25 % of the absorbance value at 280 nm)). In an effort to visualise the protein on the gel, silver staining was used (approximately 100 times more sensitive to proteins than Coomassie Blue), but the result was still negative.

7.3.2 Spectroscopic analysis of deglycosylated RfBP

Surprisingly, given the apparent lack of protein from SDS PAGE analysis of the deglycosylated sample, the CD spectrum obtained from the deglycosylated sample was not too dissimilar from the native protein (Fig. 7.1). CONTIN secondary structure analysis (Provencher and Glöckner, 1981) indicated a decrease in secondary structure.

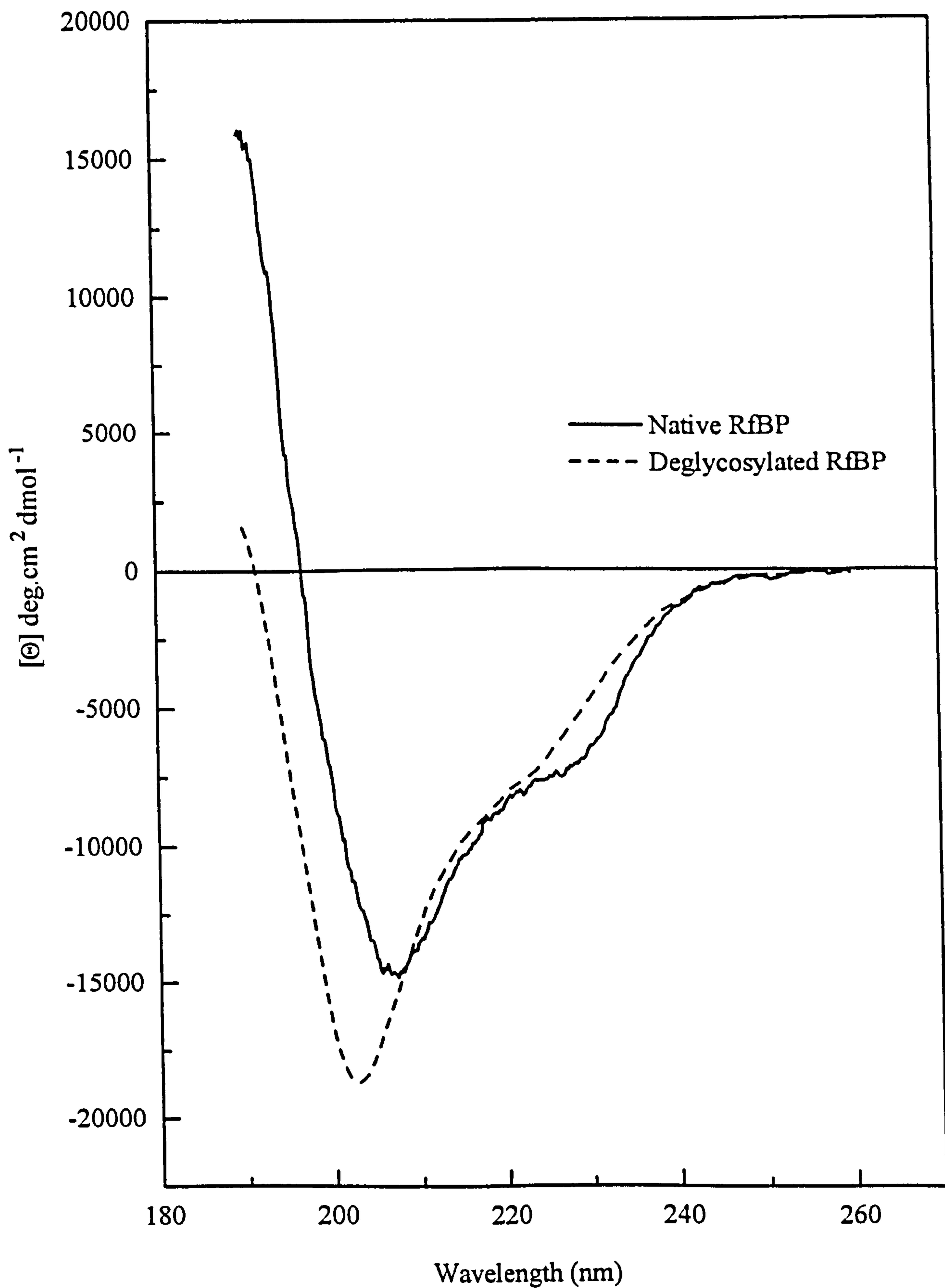


Figure 7.1 The far UV CD spectra of native and deglycosylated RfBP

From the fluorescence spectrum (Fig. 7.2), the λ_{\max} was red-shifted to 354.5 nm, indicating a virtually complete tertiary structure loss. The intensity of the peak was also greatly reduced (>50 %).

The protein was unable to bind riboflavin (Fig. 7.3).

7.4 Discussion

Fluorescence and CD were employed to determine the conformation of the protein. Although different from the native RfBP spectra, these seemed to indicate that there was protein there, albeit lacking in some secondary and tertiary structure associated with the native. The treated RfBP also failed to bind riboflavin. The TFMS incubation times (15 min to 4 hr) and temperature of the pyridine neutralisation stage (-20 to -80°C) were varied but an active deglycosylated RfBP was not produced.

The fact that a CD spectrum (although with a decreased α -helical content compared to native) was obtained, even although the Coomassie Blue stained gel seemed to suggest that there was no protein present, could indicate that the protein had fragmented into peptides big enough to form some structure, but small enough to have ran off the bottom of the gel. The TFMS neutralisation with pyridine is an extremely exothermic reaction, hence the need for precooling the reagents and maintaining a low reaction temperature. The speed of addition of the pyridine also played a part in this respect. Experimental observations demonstrated that in samples where the pyridine was added quickly, a brown precipitate was formed, and the resulting CD spectrum indicated that less secondary structure was present. Karande *et al* (1991), used the TFMS method of

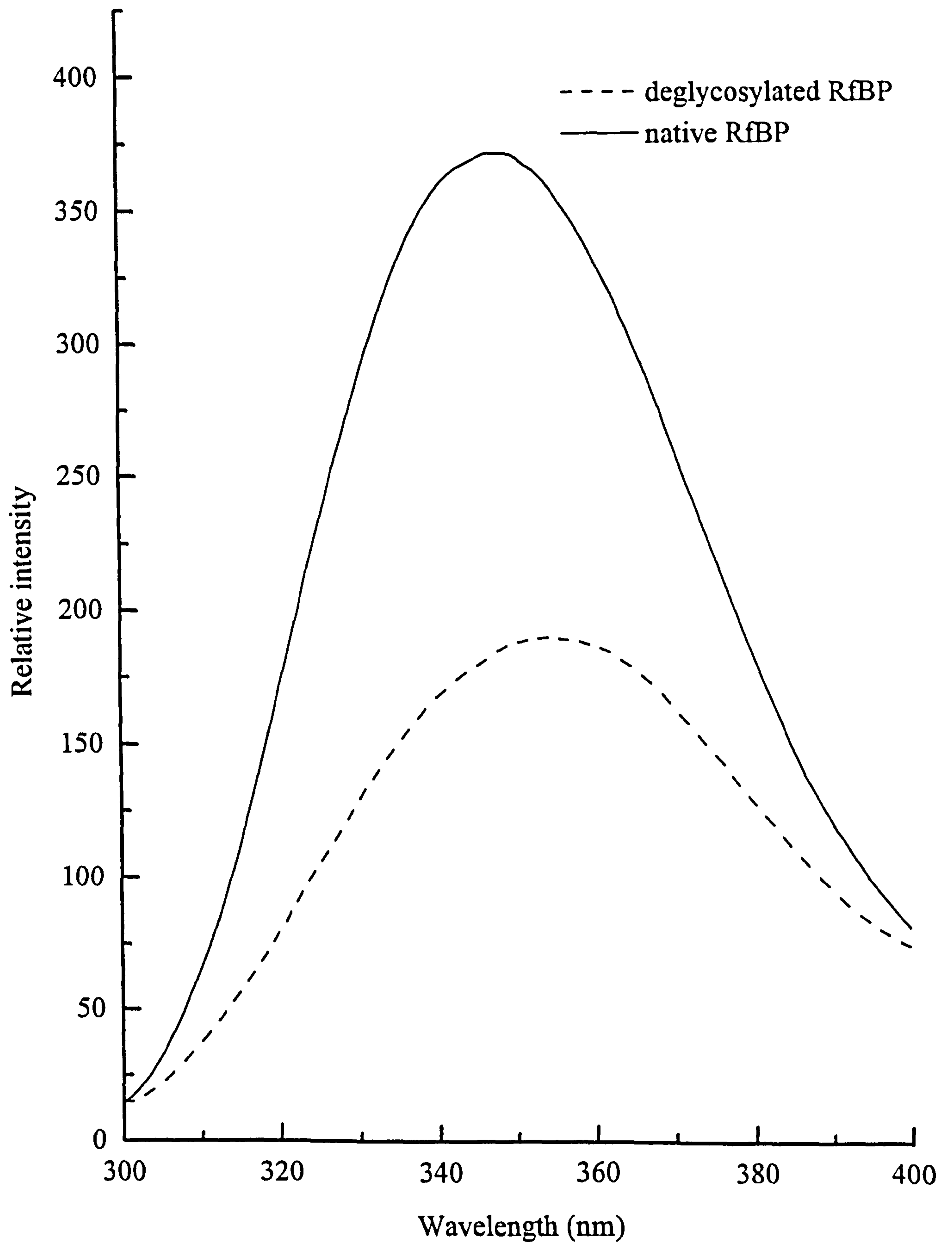


Figure 7.2 Fluorescence spectra of native and deglycosylated RfBP

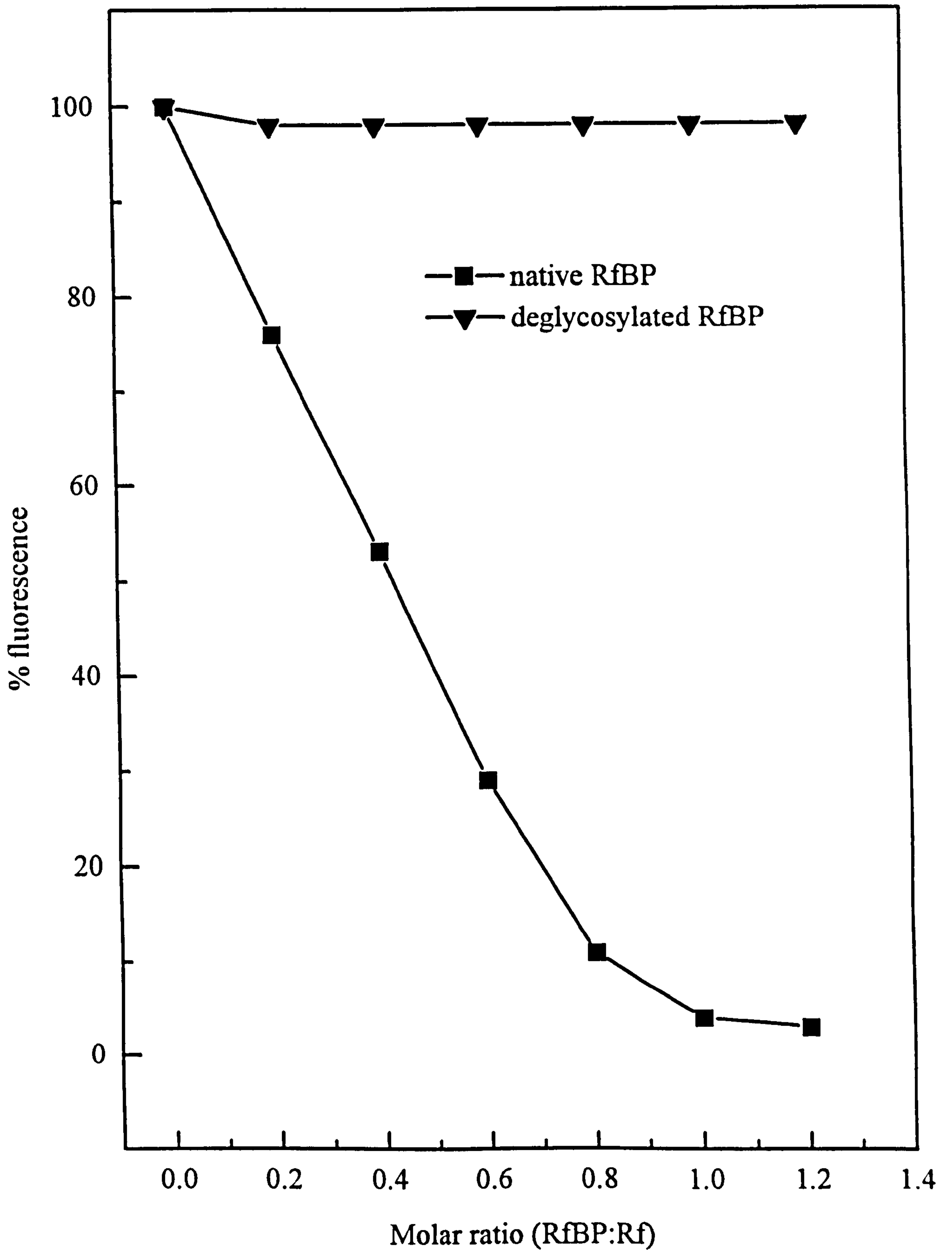


Figure 7.3 The quenching of riboflavin fluorescence by native and deglycosylated RfBP

deglycosylation on RfBP in an effort to determine the epitope of a RfBP monoclonal antibody they had developed. Although they reported that the Mab still recognised deglycosylated RfBP, through personal correspondence it was discovered that the treated RfBP was unable to bind riboflavin, and that later conformation sensitive Mabs did not recognise the treated RfBP. The epitope for the Mab that recognised the TFMS/pyridine treated RfBP was later determined to be the C-terminus, revealing that it is exposed on the surface of the protein (Velu *et al.*, 1992). As it is known that TFMS is a strong denaturant (Manjunath and Sairam, 1984), and given the extreme heat generated by the pyridine addition, coupled with the highly acidic environment, it is highly likely that chemical deglycosylation of RfBP using the Sojar and Bahl method results in denaturation and/or fragmentation of RfBP, and therefore this method is unsuitable for the production of deglycosylated RfBP in which the integrity of the polypeptide chain is preserved.

Some refolding studies on mammalian proteins that have been expressed in *E.coli* (which lack the necessary cellular machinery for glycosylation) have suggested that the absence of carbohydrates has no effect on the kinetics of successful folding (Freedman, 1992). The absence of carbohydrate groups, with the resultant change in solubility, however, may promote the aggregation of folding proteins. Yeast invertase is non-glycosylated in the cytoplasm, but the secreted protein undergoes glycosylation. Refolding studies have shown that at low concentrations, both forms of the protein will refold to a give a similar yield (Schulke and Schmid, 1988a,b). At higher concentrations, however, the cytoplasmic (non-glycosylated) protein forms aggregates. This can explain the disruption of protein secretion sometimes found upon the

perturbation of glycosylation (i.e. by tunicamycin, an antibiotic) (see Freedman, 1992, and references therein). Instead of the carbohydrates acting as a targeting signal, it is more likely that the nascent proteins aggregate, thus forming no product for secretion.

Chapter 8 General Discussion

8.1 Summary of results

Riboflavin binding protein was purified from domestic hen egg white. The purified apo-protein had identical CD and fluorescence spectra to those previously obtained, and bound riboflavin in a 1:1 molar ratio. The protein was found to be fully unfolded in 6 M Gdn HCl, with a complete loss of secondary and tertiary structure, and riboflavin binding ability. The unfolded protein was found to refold rapidly on the removal of the denaturant. The secondary structure of the protein (as measured by far UV CD) was completely regained within approximately 12 msec (the dead-time of the stopped-flow instrument in CD mode). The regain of tertiary structure of the protein (as measured by protein fluorescence) was resolved into 4 main phases, namely very fast, fast, slow and very slow. 80% of the protein fluorescence was regained within 2 msec (the dead-time of the stopped-flow instrument in fluorescence mode). The fast phase involved the regain of approximately 10% of the protein fluorescence ($t_{1/2} = 25-35$ msec), while the slow and very slow phases both represented approximately 5% of the total protein fluorescence regain, with half-times of 46 seconds and 1 hour respectively. The presence of riboflavin has no effect on the early stages of refolding, although it seemed to impede the acquisition of the final 5% of tertiary structure. The binding of riboflavin by the native protein was found to be very fast, with a half-time of approximately 3.5 msec. The refolding RfBP was able to bind riboflavin quickly ($t_{1/2} = 30$ msec) after the start of the refolding reaction, showing that the formation of the riboflavin binding pocket corresponded with the fast phase of refolding.

RfBP was found to bind ANS, resulting in a 20-fold enhancement of the ANS fluorescence. Both the apo and holo forms of the protein were able to bind ANS, but the unfolded protein was not. The binding of ANS to RfBP led to a quenching of the protein fluorescence (by 50%), and a 2 nm blueshift in the emission maximum. The refolding protein was found to regain ANS binding ability within the very fast phase (within the instrument dead-time) i.e. before the formation of the riboflavin binding site. The presence of ANS (20 μ M) had no effect on the kinetics of refolding.

RfBP contains 9 disulphide bridges. The apo-RfBP was reduced. In reoxidising conditions, the reduced protein failed to spontaneously reoxidise, instead forming random, non-native disulphide bonds. However, when protein disulphide isomerase was present, in excessive amounts, up to 50% successful reoxidation of RfBP was achieved. The presence of PDI from the start of the reaction, and low temperatures (4°C) were necessary for this reaction.

The protein contains 8 phosphorylated serine residues. A dephosphorylated protein was produced using acid phosphatase. The dephosphorylation was shown to be complete. The dephosphorylation appeared to cause very little change to the secondary and tertiary structures of RfBP. Dephosphorylation had no effect on riboflavin binding or on the refolding reactions.

Attempts to deglycosylate RfBP using TFMS were unsuccessful. It is thought that the highly exothermic nature of the reaction causes the protein to fragment.

8.2 Unfolding

Guanidinium chloride was found to readily denature RfBP, as has been found in other protein systems (see Jaenicke, 1987, and Creighton, 1990 and references therein). In 6 M Gdn HCl, RfBP exhibits all the characteristics of a random coil. Although the protein was always incubated in Gdn HCl for 15 minutes, the denaturation was very rapid (results not shown), at least within the dead-time of the steady state instrument. The process, as noted previously (Allen *et al.*, 1992), was not a simple two-state process (Section 4.3.1.2, Fig. 4.4). There appear to be two phases (0-2 M and 5-6 M Gdn HCl) where most of the changes in secondary and tertiary structure occur; a stable semi-unfolded intermediate is found between 2 and 5 M Gdn HCl. This is especially apparent from the fluorescence data, which indicates that the tertiary structure is almost constant between 2 and 5 M Gdn HCl.

A similar situation has been found with other proteins e.g. Hen egg white cystatin follows a similar pattern (Björk, 1992). In this case it was suggested that the intermediate stage represents a situation where a distinct structural element of the protein with a lower stability has been denatured independently, preceding the global unfolding of the protein. From the 3-dimensional structure available for hen cystatin, a region which could fit this hypothesis is present; a slightly flexible tight turn is present between residues Pro-72 and Met-89 - this region is visibly independent from the main body of the protein. Unfortunately, detailed structural information is not yet available for RfBP, so similar designations of structurally exposed/accessible regions cannot be made.

Other studies have noted the difference in extent of loss of secondary and tertiary structure, and activity of proteins, upon Gdn HCl denaturation (Tsou, 1986), in particular the increased susceptibility of activity/binding as compared to the overall secondary and tertiary structural features.

This is noted in RfBP, as the protein has lost all riboflavin binding ability by 2.5 M Gdn HCl, when a large proportion of secondary and tertiary structure is still present. It has been suggested that active/binding sites are situated in regions of the molecule more susceptible/accessible to denaturants; this would correspond to the exposed turn in cystatin. It is also highly possible, indeed likely, that the small changes in secondary and tertiary structure observed at low concentrations of Gdn HCl are enough to perturb the conformation of a highly structured site to a sufficient extent that specific interactions contributing to binding are lost. This slight but significant conformational change could be as a result of local denaturant activity, or part of a wider denaturant induced conformational transformation. The second possibility is indeed likely in the case of RfBP as the extremely tight binding of riboflavin is facilitated by several specific interactions between RfBP and the riboflavin molecule (Fig. 3.5).

8.3 Refolding

8.3.1 CD - regain of secondary structure

The refolding of Gdn HCl denatured RfBP was initiated by the dilution of the denaturant; this reaction was very rapid. Stopped-flow instrumentation was employed in order to attempt to resolve the refolding processes. All of the secondary structure had been regained within the dead-time of the instrument in CD mode (approx. 12

msec). This is comparable to a number of systems studied in this fashion where all, or a large proportion of the secondary structure is formed at a very early stage of refolding, within the dead-time of the stopped-flow instrumentation used (Elöve *et al.*, 1992; Kuwajima *et al.*, 1987; Kuwajima *et al.*, 1988). From these and other similar studies, it is apparent that the very rapid formation of secondary structure is a common feature in the folding of globular proteins. In fact, techniques which go beyond the time limitations of stopped-flow CD have indicated that helix formation can be extremely rapid. Refolding of short peptide sequences (which have been shown to be able to adopt native or near-native secondary structure in solution) have been used with methods such as temperature jump, electric field jump and resonant ultrasound, to study helix formation on a nanosecond time scale- this has been shown to occur in less than 160 nanoseconds (Williams *et al.* 1996). Temperature jump experiments have been carried out on barstar. Some proteins are cold-labile; at 2°C, barstar is unfolded, but a return to ambient temperature causes the protein to refold (Nölting *et al.*, 1995). This temperature jump can be achieved with a laser; a rapid phase of 3100 s⁻¹ is observed when the cold-denatured barstar is temperature-jumped from 2°C to 10°C in about 8µsec. This very rapid process involves only a small change in free energy and modest structural compaction. The compaction is followed by the major folding transition at 8 s⁻¹, detected from conventional stopped-flow techniques (Nölting *et al.*, 1995).

There is still some debate over the nature of the very early phases of protein refolding. These have been thought to be the formation of elements of local secondary structure which may act as nucleation sites for a much wider secondary structure formation and resultant hydrophobic collapse into a reasonably compact state (Mann and Matthews,

1993) However, recent studies have suggested that a non-specific hydrophobic collapse can precede any native secondary structure formation (Agashe *et al.* 1995). These processes are clearly too rapid to be measured by conventional rapid mixing techniques employed here.

8.3.2 Fluorescence - regain of tertiary structure

The refolding of RfBP as monitored via the regain of protein fluorescence revealed more of the early stages of tertiary structure acquisition. In common with other fluorescence studies, the regain of protein fluorescence consisted of a combination of fast and slower phases (Elöve *et al.*, 1992; Semisotnov *et al.*, 1987). Approximately 80% of the protein fluorescence had been regained within the dead-time of the stopped-flow instrument in fluorescence mode (< 2 msec), with only the final very slow phase taking up to 2 hours. The regain of riboflavin binding ability, which is the best indicator of successful folding, is rapid, coinciding with the fast phase (Table 4.4). This indicates that the tertiary structure at this point is very near native - the final 10% of fluorescence still to be regained at this point can only account for very minor structural changes which do not have any significant effect on binding ability.

The fact that ANS can bind to the site/sites to which it adheres at such an early stage (< 2 msec), appears to indicate that ANS binding seems to be a good test of native-like structure, which is formed very quickly. Although the ANS binding studies did not follow the classic pattern of rapid binding and slow release that would indicate the formation of a molten globule as an early intermediate in the refolding pathway, at its subsequent decay, the structure of RfBP at this point must somewhat resemble the

molten globule. The fact that ANS is not released from the refolded protein, merely confirms that the native RfBP molecule has ANS binding capability *i.e.* that hydrophobic sites/environments are present in the native molecule, and not just during refolding. Thus in this case, the apparent negative test for a molten globule state is meaningless. From this compact, condensed state, the number of possible conformations that the molecule has to explore to reach the native state is greatly reduced. This state can be said to be as good as reached upon the regain of riboflavin binding ability, after approximately 70 msec.

8.3.3 Slow phases in protein refolding

As has been discussed, with the optimisation of stopped-flow technology for the acquisition of CD and fluorescence changes on a very short time-scale, much of the recent work on protein refolding has focused on the initial very fast phases of the refolding reaction. However, it should not be forgotten that in virtually all proteins, for which the refolding process have been studied, folding proceeds via a slow stage, and that this stage, although probably much smaller in proportion to the changes observed in the fast phases, does still represent a real and measurable structural change.

The main process responsible for the long slow phases often observed in protein refolding is thought to be the isomerisation of X-Pro imide bonds (Nall, 1994). Although it is possible for the amide linkage to take either a *cis* or *trans* conformation, the *trans* form is highly favoured, and the *cis* is extremely rare (the *trans:cis* ratio is about 100-1000 to 1 in an unfolded polypeptide chain, and almost non-existent in native

protein molecules). Proline is the exception however; instead of an amide bond, they are linked by an imide bond to the preceding residue. While the imide bond still has a preference for *trans* linkages, in an unfolded structure the equilibrium distribution is 30% *cis* and 70% *trans* (Brandts *et al.*, 1975), and while still not common, *cis* imide linkages are found more frequently in native proteins than *cis* amide links (about 6 % of imide linkages are in the *cis* conformation (see Mucke and Schmid, 1992, and references therein)).

When a native protein is unfolded, any *cis* X-Pro linkages are more than likely to assume the *trans* conformation (the 30:70 split). Upon refolding, the polypeptide chain must have the correct X-Pro linkages to be able to assume the native conformation. If the imide bond exhibits the incorrect stereochemistry then it must undergo isomerisation before the refolding reaction can be completed, and the final, native tertiary structure assumed.

It was first proposed by Brandts *et al.*(1975) that the rate-limiting step in protein refolding was the *cis-trans* isomerisation of non-native proline peptide bonds; and that the slow phase in protein folding was due to a sizeable proportion of the refolding molecules having proline residues in the incorrect conformation. In some of the most commonly studied refolding systems, RNase A, ribonuclease T1 and thioredoxin all have at least one *cis* imide linkage, while lysozyme and cytochrome *c* have all *trans* linkages. The refolding kinetics of those with *cis* linkages all contain slow stages, while the pathways of those with all *trans* involve predominantly fast kinetics.

Given that the half-life of proteins *in vivo* in many cases can be shorter than the length of some slow phases found in protein refolding (*i.e.* > 30 minutes), it was likely that a system existed *in vivo* to facilitate the catalysis of *cis/trans* proline isomerisation. Peptidyl prolyl *cis-trans* isomerase (PPIase) was first purified by Fischer *et al.*, (1984) from porcine kidney. It was shown to catalyse the isomerisation of proline imide peptide bonds in oligopeptides, and later in a number of studies on protein refolding, in which slow folding phases had been observed (Lang *et al.*, 1987). In various proteins whose refolding involve slow phases, the rates of these phases have been considerably increased by the action of PPIase (Schönbrunner *et al.*, 1991a; Mucke and Schmid, 1992), demonstrating that slow phases observed in protein refolding are due solely to *cis/trans* proline linkage isomerisation (Lang *et al.*, 1987).

PPIase activity has been associated with the lumen of the ER (as well as the cytosol), the cellular compartment where folding occurs (Bose and Freedman, 1994). This compartmentalisation would localise PPIase along with PDI (Section 5.1.3) the other catalyst of protein folding. *In vitro* studies have shown that the presence of PPIase greatly increases the efficiency of PDI (Schönbrunner and Schmid, 1992). It is very possible that the two 'foldases' act in conjunction with each other, or are even mutually interdependent on each other (Schönbrunner and Schmid, 1992) (*i.e.* the isomerisation of imide linkages into their native conformation improves accessibility, or presents a more attractive site of action for PDI, and the formation of the correct disulphide linkages plays a similar role for PPIase activity.)

8.4 The effect of post-translational modifications on the refolding of RfBP, and relevance to *in vivo* folding

Of the post-translational modifications that were originally planned to be studied in this project, disulphide bond formation, and phosphorylation were investigated, while attempts to deglycosylate RfBP were unsuccessful. Given that the refolding of reduced RfBP was only possible in the presence of PDI, and the large number of disulphide linkages in RfBP, it does seem almost certain that PDI has a role in the *in vivo* folding of RfBP. The high concentration of PDI necessary for a reasonable level of successful reoxidation (to a native-like state) is consistent with the high levels of PDI present in the lumen (thought to be in the millimolar range).

Dephosphorylation of RfBP seemed to have absolutely no effect whatsoever on the refolding kinetics of RfBP. Although it might have been expected that the removal of eight, closely grouped negatively charged groups might have had some effect on the refolding protein (e.g. through reduced electrostatic interactions), this was not found. The phosphorylated serines are found towards the end of the polypeptide chain, and the accessibility of the phosphates to phosphatase suggests that they are on the outside of the molecule; it could be that this portion of the protein is only really involved in the folding process towards the end of the reaction, and has little interaction with the rest of the molecule. Certainly, the fact that, *in vivo*, phosphorylation probably occurs after folding has been completed, would imply that phosphorylation does not play a crucial role in determining the protein folding process.

8.5 Future work

The fast phases of the refolding process have been well characterised in this study. The slow and the very slow phases observed are thought to be due to uncatalysed *cis/trans* proline isomerisation. RfBP has 8 proline residues. To verify whether any of them exist in the *cis* conformation, a high resolution 3-D structure of RfBP would be required, and to date, this information is not yet available. If the rate of the 2 slow phases were increased by refolding in the presence of PPIase, then this would confirm the proline isomerisation theory for the slow phases. Although the amplitude of these phases is small, and a change in the kinetics of these might be hard to detect, the effect of PPIase can be quite significant (Lang *et al.*, 1987; Schönbrunner *et al.* 1991). It is hoped that a sample of PPIase will be obtained in the near future, and that this experiment will be undertaken.

A 3-D structure for RfBP has still not been resolved. Zanette *et al.* (1984) reported obtaining crystals; unfortunately these did not diffract sufficiently, and attempts at producing isomorphous replacements were unsuccessful (personal correspondence with L. Sawyer). A detailed structure would surely provide greater insights into the refolding of RfBP (conformation of imide links etc.), and also the mode of binding of riboflavin to RfBP, with the resultant quenching of riboflavin fluorescence.

The deglycosylation of RfBP by chemical means (TFMS acid) was unsuccessful. Future attempts to deglycosylate RfBP could involve the use of endoglycosylases, which enzymatically cleave carbohydrates from polypeptides. Deglycosylated RfBP produced through enzyme action could be used to investigate the role of glycosylation in the

refolding of RfBP.

Appendix 1

Estimation of the Dead-time for the Stopped-flow Instrument

The estimation of the dead-time of the SX.17MV stopped-flow spectrophotometer was performed as according to Tonomura *et al.* (1977). Their method uses the colour change induced by the reduction of 2,6-dichlorophenolindophenol (DCPIP) by L-ascorbic acid.

The following solutions were prepared:

i) Hydrochloric acid (0.02 M)/sodium chloride (0.2 M)

100 ml of 0.1 M HCl + 5.8 g of NaCl diluted to 500 ml with double distilled H₂O.

ii) DCPIP (500 µM)

14.5 mg of DCPIP dissolved in 10 ml of propanol +
1.16 g NaCl diluted to 100 ml with double distilled H₂O.

iii) Stock ascorbic acid solution (200 mM)

3.52 g ascorbic acid diluted to 100 ml with HCl/NaCl solution.

iv) Working ascorbic acid solutions

Working solutions of 5, 10, 20, 50 and 100 mM were prepared from the stock solution by dilution with the HCl/NaCl solution.

With the instrument in absorbance mode, the absorbance change at 524 nm was measured over 50 msec. The reaction was followed with the mixing in 1:1, and the 1:10 mode (the concentration of ascorbic acid (in the small syringe) was increased). 10 traces were averaged, and the kinetic data at each concentration was analysed. Data analysis was restricted to data collected from 2 msec after the triggering of the machine.

The dead-time for each reaction was calculated using the reaction :

$$\frac{t_d}{t_{1/2}} = \frac{\ln (X_{tot}/X_{obs})}{\ln 2} ,$$

where t_d = dead-time,

$t_{1/2}$ = half-time

X_{tot} = theoretical total initial absorbance

X_{obs} = observed initial absorbance

For 1:1 mixing, the dead-time was found to be 2.045 msec \pm 0.114 (n=10), and for the 1:10 mixing (used in refolding reactions), 1.733 msec \pm 0.176 (n=10). This was found to correspond reasonably with the manufacturers' estimates (1.34-1.37 msec).

The dead-time for the instrument in CD mode was not calculated due to hardware difficulties. However, the manufacturer estimates that the dead-time for the instrument

in CD configuration (1:10 mixing) is approximately 12 msec. The longer dead-time (compared to the instrument in absorbance/fluorescence mode) is due to the different dimensions of the CD cell.

References

- AGASHE, V.R., SHASTRY, M.C.R. and UDGAONKAR, J.B. (1995) *Nature* 377: 754-757
- ALEXANDER, S., HUBBARD, S.C. and STROMINGER, J.L. (1984) *J. Immunol.* 133: 315-320
- ALLEN, S.A., STEVENS, L., DUNCAN, D., KELLY, S.M. and PRICE, N.C. (1992) *Int. J. Biol. Macromol.* 14: 333-337
- AMES, B.N. and DUBIN, D.T. (1959) *J. Biol. Chem.* 235: 769-775
- ANDRIA, G. and TANIUCHI, H. (1978) *J. Biol. Chem.* 253: 2262-2270
- ANFINSEN, C.B. (1973) *Science* 181: 223-230
- AOKI, T., YAMAO, Y., YONEMASU, E., KUMASAKI, Y. and KAKO, Y. (1993) *Arch. Biochem. Biophys.* 305: 242-246
- BEVCAR, J. and PALMER, G. (1982) *J. Biol. Chem.* 257: 5607-5617
- BJÖRK, I. and POL, E. (1992) *FEBS Lett.* 299: 66-68
- BLANKENHORN, G. (1978) *Eur. J. Biochem.* 82: 155-160
- BOSE, S. and FREEDMAN, R.B. (1994) *Biochem. J.* 300: 865-870
- BRANDTS, J.F., HALVORSON, H.R. and BRENNAN, M. (1975) *Biochemistry* 14: 4953-4963
- BREATHNACH, R., MANDEL, J.L. and CHAMBOU, P. (1977) *Nature* 270: 314-319
- BULLEID, N.J. and FREEDMAN, R.B. (1988) *Nature* 335: 649-651
- BYCROFT, M., MATOUSCHEK, A., KELLIS, J.T., Jr., SERRANO, L. AND FERSHT, A.R. (1990) *Nature* 346: 488-490
- CANDY, D.J. (1980) *Biological Functions of Carbohydrates* Blackie

- CHOI, J.-D. and MCCORMICK, D.B. (1980) *Arch. Biochem. Biophys.* 204: 41-51
- CHOU, P.Y. and FASMAN, G.D. (1978) *Ann. Rev. Biochem.* 47: 251-276
- CREIGHTON, T.E. (1990) *Biochem. J.* 270: 1-16
- CREIGHTON, T.E. (1994) in *Mechanisms of Protein Folding* pp. 1-25 (Ed. Pain, R.H.) I.R.L. Press
- CREIGHTON, T.E. (1994a) *Struct. Biol.* 1: 135-138
- CUTTING, J.A. (1984) *Methods. Enzymol.* 104: 451-453
- DARBY, N.J. and CREIGHTON, T.E. (1993) *Protein Structure* I. R. L. Press
- DOBSON, C.M., EVANS, P.A. and RADFORD, S.E. (1994) *Trends Biochem. Sci.* 19: 31-34
- DODD, G.H. and RADDA, G.K. (1969). *Biochem. J.* 114: 407-417
- EDGE, A.S.B., FALTYNEK, C.R., HOF, L., REICHERT, L.E. AND WEBER, P. (1981) *Anal. Biochem.* 118: 131-137
- ELLMAN, G.L. (1959) *Arch. Biochem. Biophys.* 82: 70-77
- ELÖVE, G.A., CHAFFOTTE, A.F., RODER, H. and GOLDBERG, M.E. (1992) *Biochemistry* 31: 6876-6883
- ENGELHARD, M. and EVANS, P.A. (1995) *Protein Sci.* 4: 1553-1562
- EPSTEIN, C.J., GOLDBERGER, R.F. and ANFINSEN, C.B. (1963) *Cold Spring Harbour Symp. Quant. Biol.* 28: 439-449
- FERSHT, A. (1985) *Enzyme Structure and Function* (2nd Edition) W.H. Freeman and Co.
- FISCHER, G., BANG, H. and MECH, C. (1984) *Biomed. Biophys. Acta* 43: 1101-1111
- FREEDMAN, R.B., BROCKWAY, B.E. and LAMBERT, N. (1984) *Biochem. Soc. Trans.* 12: 929-932

- FREEDMAN, R.B., BULLEID, N.J., HAWKINS, H.C. and PAVER, J.L. (1989) **Biochem. Soc. Symp.** 55: 167-192
- FREEDMAN, R.B. (1989) **Cell** 57: 1069-1072
- FREEDMAN, R.B. (1992) in **Protein Folding** (Ed. Creighton, T.E.) W. H. Freeman and Co.
- FREEDMAN, R.B., HIRST, T.R., TUIITE, M.F. (1994) **Trends Biochem. Sci.** 19: 331-336
- FREEDMAN, R.B., HAWKINS, H.C. AND McLAUGHLIN, S.H. (1995) **Methods Enzymol.** 251: 397-406
- FREIFELDER, D. (1982) **Physical Biochemistry** (2nd edition) W. H. Freeman and Co.
- FRUTON, J.S. (1972) **Molecules of Life** (1st Edition) Wiley-Interscience
- GILBERT, A.B. (1971) **Physiology of the Domestic Fowl** pp. 1379-1409 (Eds. Bell, D.J. and Freeman, B.M.), Academic Press
- GILBERT, H.F. (1989) **Biochemistry** 28: 7298-7305
- GIVOL, D., GOLDBERGER, R.F. and ANFINSEN, C.B. (1964) **J. Biol. Chem.** 239: 3114-3116
- GOLDBERGER, R.F., EPSTEIN, C.J. and ANFINSEN, C.B. (1963) **J. Biol. Chem.** 238: 628-635
- GOTTLIEB, M. and CHAVKO, M. (1987) **Anal. Biochem.** 165: 33-37
- HAMAZUME, Y., MEGA, T., IKENAKA, T. (1984) **J. Biochem.** 95: 1633-1644
- HAMAZUME, Y., MEGA, T., IKENAKA, T. (1987) **J. Biochem.** 101: 217-23
- HAMMES, G.G. (1982) **Enzyme Catalysts and Regulation** Academic Press.
- HARRIS, D.A. and BASHFORD, C.L. (Eds.) (1987) **Spectrophotometry and Spectrofluorimetry: a Practical Approach** Oxford Press

- HARRISON, S.C. and DURBIN, R. (1985) *Proc. Natl. Acad. Sci. USA* 82: 4028-4030
- HILLSOM, D.A., LAMBERT, N. and FREEDMAN, R.B. (1984) *Methods Enzymol.* 107: 281-294
- HSIEH, P., ROSNER, M.R. and ROBBINS, P.W. (1983) *J. Biol. Chem.* 258: 2548-2554
- IMOTO, T., JOHNSON, L.N., NORTH, A.C.T., PHILLIPS, D.C. AND RUPLEY, J.A. (1972) in *The Enzymes* (3rd Edition) (Ed. Boyer, P.B.)
- JAENICKE, R. (1987) *Prog. Biophys. Mol. Biol.* 49: 117-237
- JONES, B.E., BEECHAM, J.M., and MATTHEWS, C.R. (1995) *Biochemistry* 34: 1867-1877
- KANE, J.F. and HARTLEY, D.L. (1988) *Trends Biotechnol.* 6: 95-101
- KARANDE, A.A., VELU, N.R., ADIGA, P.R. (1991) *Mol Immunol.* 28: 471-478
- KOIVU, J., MYLLYLA, R., HELAAKOSKI, T., PIHLAJANIEMI, T., TASANEN, K. and KIVIRIKKO, K.I. (1987) *J. Biol. Chem.* 262: 6447-6449
- KOZIK, A. (1982) *Biochim. Biophys. Acta* 704: 542-545
- KOZIK, A. (1982a) *Eur. J. Biochem.* 121: 395-400
- KRISHAMURTHY, K., SWOLIA, N. and ADIGA, P.A. (1984) *FEBS Lett.* 178: 87-91
- KUMOSINSKI, T.F., PESSEN, H. and FARRELL, H.M.Jr. (1982) *Arch. Biochem. Biophys.* 214: 714-725
- KUWAJIMA, K., YAMAYA, S., MIWA, S., SUGAI, S. and NAGAMURA, T. (1987) *FEBS Lett.* 221: 115-118
- KUWAJIMA, K., SAKURAOKA, A., FUEKI, S., YONEYAMA, M. and SUGAI, S. (1988) *Biochemistry* 27: 7419-7428
- LAEMMLI, U.K. (1970) *Nature* 227: 680-685

- LANG, K., SCHMID, F.X., FISCHER, G. (1987) *Nature* 329: 268-270
- LEE, C.M. and WHITE, H.B. (1995) *J. Nutr.* 126: 523-528
- LIS, H. and SHARON, N. (1993) *Eur. J. Biochem.* 218: 1-27
- LYLES, M.M. and GILBERT, H.F. (1991) *Biochemistry* 30: 619-625
- MacLACHLAN, I., NIMPF, J., WHITE, H.B. and SCHNEIDER, W.J. (1993) *J. Biol. Chem.* 268: 23222-23226
- MacLACHLAN, I., NIMPF, J. and SCHNEIDER, W.J. (1994) *J. Biol. Chem.* 302: 24127-24132
- McCLELLAND, D.A., McLAUGHLIN, S.H., FREEDMAN, R.B. and PRICE, N.C. (1995) *Biochem. J.* 311: 133-137
- McLAUGHLIN, A.C. (1974) *J. Biol. Chem.* 240: 1445-1452
- MANJUANATH, P. and SAIRAM, M.R. (1984) *Methods Enzymol.* 109: 725-735
- MANN, C.J. and MATTHEWS C.R. (1993) *Biochemistry* 32: 5282-5290
- MARSTON, F.A.O. (1986) *Biochem. J.* 240: 1-12
- MATTHEWS, C.R. (1993) *Ann. Rev. Biochem.* 62: 653-683
- MEGA, T., HAMAZUME, Y., NONG, Y.M. and IKENAKA, T. (1985) *J. Biochem.* 60: 1109-1116
- MILLER, M.S., BUSS, E.G. and CLAGETT, C.O. (1981) *Biochim. Biophys. Acta* 677: 225-233
- MILLER, M.S., BENMORE-PARSONS, M. and WHITE, H.B. (1982) *J. Biol. Chem.* 257: 6818-6824
- MILLER, M.S., BRUCH, R.C. and WHITE, H.B. (1982a) *Biochim. Biophys. Acta* 715: 126-136
- MILLER, M.S. and WHITE, H.B. (1986) *Methods Enzymol.* 122: 227-234

- MURRY-BRELIER, A., and GOLDBERG, M. (1989) **Prot. Struct. Funct. Genet.** 6: 395-404
- MÜCKE, M. and SCHMID, F.X. (1992) **Biochemistry** 31: 7848-7854
- NALL, B.T. (1994) in **Mechanisms of Protein Folding** pp. 80-103 (Ed. Pain, R.H.) I.R.L. Press
- NISHIKIMI, M. and KYOGOKU, Y.J. (1973) **J. Biochem.** 73: 1233-1242
- NÖLTING, B., GOLBIK, R. and FERSHT, A.R. (1995) **Proc. Natl. Acad. Sci. USA** 92: 10668-10678
- NORIOKA, N., OKADA, T., HAMAZUME, Y., MEGA, T. and IKENAKA, T. (1985) **J. Biochem.** 97: 19-28
- NOZAKI, Y. (1972) **Methods Enzymol.** 26: 43-50
- OHGUSHI, M. and WADA, A. (1983) **FEBS Lett.** 164: 21-24
- PETERS, T. and DAVIDSON, L.K. (1982) **J. Biol. Chem.** 256: 8847-8853
- PHILLIPS, D.C. (1967) **Proc. Natl. Acad. Sci. USA** 57: 484-495
- PRICE, N.C. (1995) in **Molecular Biology and Biotechnology** V.C.H Publishers, Inc.: 179-185
- PRICE, N.C. AND STEVENS, L. (1989) **Fundamentals of Enzymology** (2nd edition) Oxford University Press
- PROVENCHER, S.W. and GLÖCKNER, J. (1981) **Biochemistry** 20: 33-37
- PTITSYN, O.B., PAIN, R.H., SEMISOTNOV, G.V., ZEROVNIK, E. and RAZGULYAOV, O.I. (1990) **FEBS Lett.** 262: 20-24
- PTITSYN, O.B. (1992) in **Protein Folding** pp 243-300 (Ed. Creighton, T.E.) W.H. Freeman and Co.
- RADFORD, S.E., DOBSON, C.M. and EVANS, P.A. (1992) **Nature** 358: 302-307

RHODES, M.B., AZARI, P.R. and FEENEY, R.E. (1958) *J. Biol. Chem.* 230: 399-408

RHODES, M.B., BENNETT, N. and FEENEY, R.E. (1959) *J. Biol. Chem.* 234: 2054-2060

RICHARDSON, J.S. and RICHARDSON, D.C. (1989) *TIBS* 14: 304-309

ROTH, R.A. and PIERCE, S.B. (1987) *Biochemistry* 26: 4179-4182

ŠALI, A., SHAKHNOVICH, E. and KARPLUS, M. (1994) *Nature* 369: 248-251

SARGENT, J.R. and GEORGE, S.G. (1975) *Methods in Zone Electrophoresis* B.D.H. Chemicals, Poole.

SCHMID, F.X. (1992) in *Protein Folding* (Ed. Creighton, T.E.) W.H. Freeman and Co.

SCHÖNBRUNNER, E.R., MAYER, S., TROPSCHUG, M., FISCHER, G., SEMISOTNOV, G.V., RODIONOVA, N.A., RAZGULAOV, O.I. UVERSY, V.N., GRIPAS A.F. and GILMANSHIN, R.I. (1991) *Biopolymers* 37: 119-128

SCHÖNBRUNNER, E.R., MAYER, S., TROPSCHUG, M., FISCHER, G., TAKAHASHI, N. and SCHMID, F.X. (1991a) *J. Biol. Chem.* 266: 3630-3635

SCHÖNBRUNNER, E.R. and SCHMID, F.X. (1992) *Proc. Natl. Acad. Sci. USA* 89: 4510-4513

SCHÜLKE, N. and SCHMID, F.X. (1988a) *J. Biol. Chem.* 263: 8827-8831

SCHÜLKE, N. and SCHMID, F.X. (1988b) *J. Biol. Chem.* 263: 8832-8837

SEMISOTNOV, G.V., RODIONOVA, N.A., KUTYSHENKO, V.P., EBERT, B., BLANCK, J. and PTITSYN, O.B. (1987) *FEBS Lett.* 224: 9-13

SHORTLE, D. and MEEKER, A.K. (1989) *Biochemistry* 28: 934-944

SOJAR, H.T. and BAHL, O.P. (1987) *Methods Enzymol.* 138: 341-350

STEVENS, L. (1991) *Comp. Biochem. Physiol.* 100B:1-9

STEVENS, L. (1996) *Science Progress* 79: 65-87

STRYER, L. (1988) **Biochemistry** (3rd Edition) W.H.Freeman and Co., New York

TAKAHASHI, N. and SCHMID, F.X. (1991) **J. Biol. Chem.** 266: 3630-3635

TANFORD, C. (1968) **Adv. Prot. Chem.** 23: 121-282

TANIUCHI, H. (1978) **J. Biol. Chem.** 245: 5459-5468

TONOMURA, B., NAKATANI, H., MASATAKE, O., JUNKO, Y-I. and HIROMI, K. (1977) **Anal. Biochem.** 84: 370-383

TSONG, T.Y. (1976) **Biochemistry** 15: 5467-5473

TSOU, C.-L., (1986) **TIBS** 11: 427-429

VELU, N.K., KARANDE, A.A. and ADIGA, P.R. (1992) **J. Biosci.** 17: 151-165

VISWESWARIAH, S.S. and ADIGA, P.R. (1987) **Bioscience Reports** 7: 563-571

VUORI, K., PIHLAJANIEMI, T., MYLLYLA, R., KIVIRIKKO, K.I. (1992) **EMBO J.** 11: 4213-4217

WALKER, M., STEVENS, L., DUNCAN, D., PRICE, N.C. and KELLY, S.M. (1991) **Comp. Biochem. Physiol.** 100B: 77-81

WATSON, J.D., HOPKINS, N.H., ROBERTS, J.W., STEITZ, J.A. and WEINER, A.M. (1987) **Molecular Biology of the Gene** (4th Edition) The Benjamin/Cummings Publishing Co, Inc.

WETTERAU, J.R. and COMBS, K.A., SPINNER, S.N., JOINER, B.J. (1990) **J. Biol. Chem.** 265: 9800-9807

WHITE, H.B. and MERRILL, A.H. (1988) **Ann. Rev. Nutr.** 8: 279-299

WHITE, H.B. (1990) **USA Today** September: 64-66

WILLIAMS, S., CAUSGROVE, T.P., GILMANSHIN, R., FANG, K.S., CALLENDER, R.H., WOODRUFF, W.H., DYER, R.B. (1996) **Biochemistry** 35: 691-697

WINTER, W.P., BUSS, E.G., CLAGETT, C.O. and BOUCHER, R.V. (1967) **Comp.**

Biochem. Physiol. 22: 897-906

**WUNDERLICK, M., OTTO, A., MASKOS, K., MÜCKE, M., SECKLER, R. and
GLOCKSHUBER, R. (1995) J. Mol. Biol. 247: 28-33**

**ZANETTE, D., MONACO, H.L., ZANOTTI, G. and SPADON, P. (1984) J. Mol. Biol.
180: 1185-1187**

**ZHENG, D.B., LIM, H.M., PENE, J.J. and WHITE, H.B. (1988) FASEB J. 263, 11126-
11129**

**ZWANZIG, R., SZABO, A. and BAGCHI, B. (1992) Proc. Natl. Acad. Sci. USA 89,
20-22**