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CHARACTERIZATION OF THE TERTIARY STRUCTURE OF THE *DE NOVO* DESIGNED PROTEIN MB-1

A Thesis

submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Jillian D. MacCallum

Charlottetown, P.E.I.

September, 1997

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ABSTRACT

Milk Bundle-1 (MB-1) is a *de novo* designed protein with 100 amino acids, having a molecular weight of 11.4 kilodaltons. MB-1 is enriched with 57% of selected essential amino acids (methionine, threonine, lysine and leucine), which are known to be limiting in dairy cattle. Recently, on the basis of a digestibility study, MB-1 was predicted to be unstable in rumen conditions. Such a property would decrease MB-1 value as an ideal protein for lactating cows. In order to understand the reasons for MB-1's limited stability, characterization of its tertiary structure was undertaken.

Characterization of the protein's structure was achieved using fluorescence spectroscopy (steady state measurements). MB-1 contains one tyrosine at position 62, expected to be in position "d" of helix III, in the hydrophobic core. Data obtained using fluorescence quenching indicates that the tyrosine is protected from the solvent in the putative hydrophobic core, as per design.

Once it was established that MB-1 was not misfolded, further experiments were done to assess the fluidity of its hydrophobic core. For this, the amphiphillic dye ANSA was used. Results obtained for MB-1 compare favourably to those of many natural proteins, suggesting that MB-1 has achieved some degree of nativeness. Interestingly, MB-1 was found to exclude ANSA from its hydrophobic core more efficiently than all other *de novo* designed proteins reported to date.

Finally, an analysis of folding thermodynamics of MB-1 was attempted. It was found that the fluorescence intensity of tyrosine was not sensitive to unfolding, making thermodynamic data impossible to obtain. By adding citrate as a quencher during unfolding it was possible to detect cold denaturation of MB-1 above 0 °C, as a rare phenomenon. This approach seems to be suitable and should allow for a complete thermodynamic analysis in the future.

Analysis of the data on MB-1 as compared to other natural proteins indicates that MB-1 is folded and compact. The lack of resistance to proteases must be caused by other factors other than the lack of compactness or misfolding. Future mutations to the protein, such as fold stabilizers, would increase the proteins conformational stability and therefore it's stability in the rumen environment.

ACKNOWLEDGMENTS

I would like to thank first and foremost my supervisor, Dr. Marc Beauregard, for his support, patience, guidance and understanding throughout my Master of Science program and mostly for believing in me, without which none of this would have been possible. Special thanks to Dr. Liz Wirtanen and Dr. Jean Grundy for their excellent advice and guidance. Also special thanks to Jennifer Morrison. I also extend my appreciation to members of my supervisory committee, Drs. F. Markham, F. Kibenge, and M.S. Nijjar.

I am grateful to the faculty and staff of Departments of Chemistry, and Pathology and Microbiology, UPEI. The technical help of Mrs. Dawna Lund was greatly appreciated. Last but not least I thank my family, friends, and loved ones for their love, patience and moral support during the last two years. I owe you all a great deal.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

COMMONLY USED ABBREVIATIONS

TERM	ABBREVIATION
Essential amino acids	EAA
Milk Bundle -1	MB-1
Methionine	M
Threonine	T
Lysine	K
Leucine	L
Tyrosine	Y

Standard units of measurements

degree Celsius	°C
centrifugal force	g
kilodalton	kDa
millilitre	mL
millimolar	mM
molecular weight cut off	MWCO
microlitre	µL
molar	M
microgram	µg
nanometre	nm
maximum wavelength	λ_{max}
hydrogen ion activity	pH
percent	%
hours	hr
photons/sec	p/s

Chemicals

ethylenediaminetetracacetate	EDTA
sodium hydroxide	NaOH
isopropyl β-D-thiogalacto-pyranoside	IPTG

phenylmethylsulfonlyfluoride	PMSF
ribonuclease A	RNAse
cytochrome *c*	cyt. *c*
maltose binding protein	MBP
sodium dodecyl sulphate	SDS
polyacrylamide gel electrophoresis	PAGE
diethylaminoethyl	DEAE
8-anilino-1-napthalene sulphonic acid	ANSA

1. Introduction

1.1 Protein Structure

Proteins and peptides are the main components of the biomass of cells. The transfer of information in cells from gene to protein is a complex process. The information contained in DNA is transcribed into mRNA and then translated into a linear sequence made up of amino acids. All twenty amino acids have a general structure in common with variations in their side chains. Each amino acid has an amino group and a carboxylic acid group. Both of these groups are bonded to the same carbon, the α-carbon. This α-carbon is also bonded to a side chain group (termed an R-group) which differs with each amino acid (Figure 1).

Based on the nature of their side chains, amino acids fall into three groups: polar, non-polar and charged (Campbell, 1991).

The linear amino acid sequence of a polypeptide represents its primary structure. Different regions of this sequence form local regular secondary structures, such as alpha helices or beta strands. Secondary structure refers to 3-D structure, and is characterized by defined hydrogen bond networks. The tertiary structure is formed by packing such structural elements into one or several compact globular units called domains (Figure 2). Both the secondary and tertiary structure are mostly determined by the properties of the amino acid sequence.

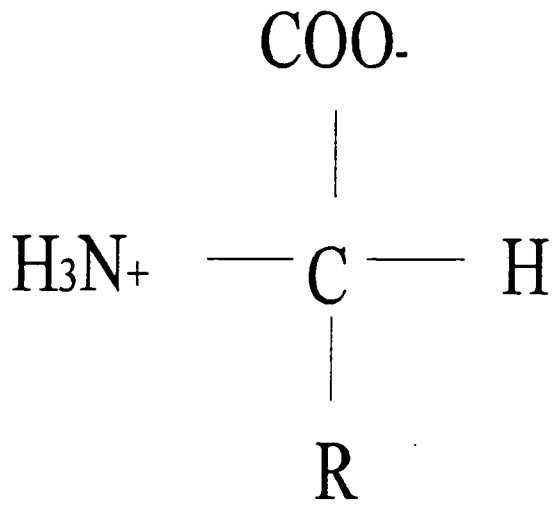
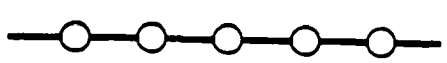
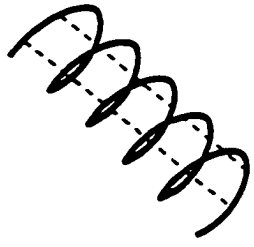


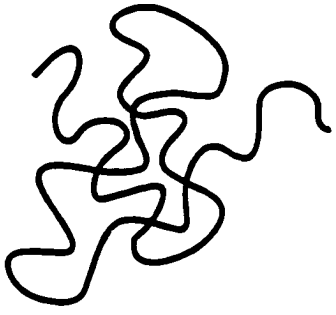
Figure 1: The general formula of amino acids showing the ionic forms predominant at pH 7.0.



Primary Structure



Secondary Structure



Tertiary Structure

Figure 2: Hierarchy of Protein Structure. Synopsis of the increasingly complex arrangement of structural elements from the amino acids of the primary sequence to the folding patterns of the secondary and tertiary folds. The linear sequence of amino acids defines the primary structure; secondary structures consists of regions of regularly repeating conformations of the peptide chain such as α-helix (dashe dlines represent hydrogen bonds) ; tertiary structure describes the shape of the fully folded polypeptide chain.

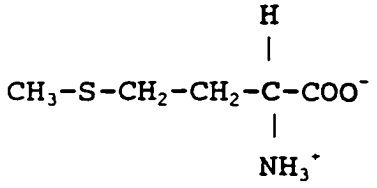
Of the twenty amino acids found in nature, nine of them are termed essential amino acids because they cannot be synthesized by animal eukaryotic cells and must be taken in through the diet, (some of them are shown in figure 3). Prokaryotic cells, however, can synthesize all twenty amino acids. Some essential amino acids are: lysine, methionine, phenylalanine, threonine, leucine, tryptophan, and valine (Campbell, 1991).

1.2 Protein and the Dairy Cow:

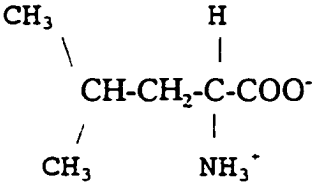
1.2.1 Digestion and Absorption

The absorption of essential amino acids (EAA) from digested protein is vital to the maintenance, reproduction, growth, and lactation of the dairy cow . The essential amino acids must come from either dietary (mainly plant) protein that escapes rumen degradation or from rumen microbial protein. The major sources of protein for cows are plants and rumen microorganisms. Plant source is usually from forages, concentrates and protein supplements such as the oilseed meals (Miller, 1979). Of the high-protein supplementary feeds, the one used in greatest amount is soybean meal, which contains on average 45-50% of crude protein (Cullision, 1975).

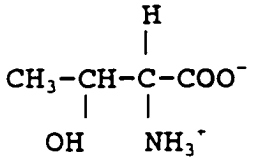
Cows also get their protein from rumen microbes. Rumen microbes synthesize their own protein from the amino acids and ammonia released by degradation of the crude protein from feed, non-protein nitrogen and sulphur present in the rumen (Figures 4a, 4b) (Chalupa, 1975).



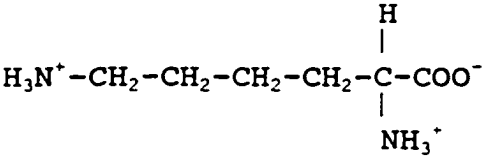
Methionine



Leucine



Threonine



Lysine

Figure 3: The structure of the essential amino acids methionine, threonine, lysine , and leucine.

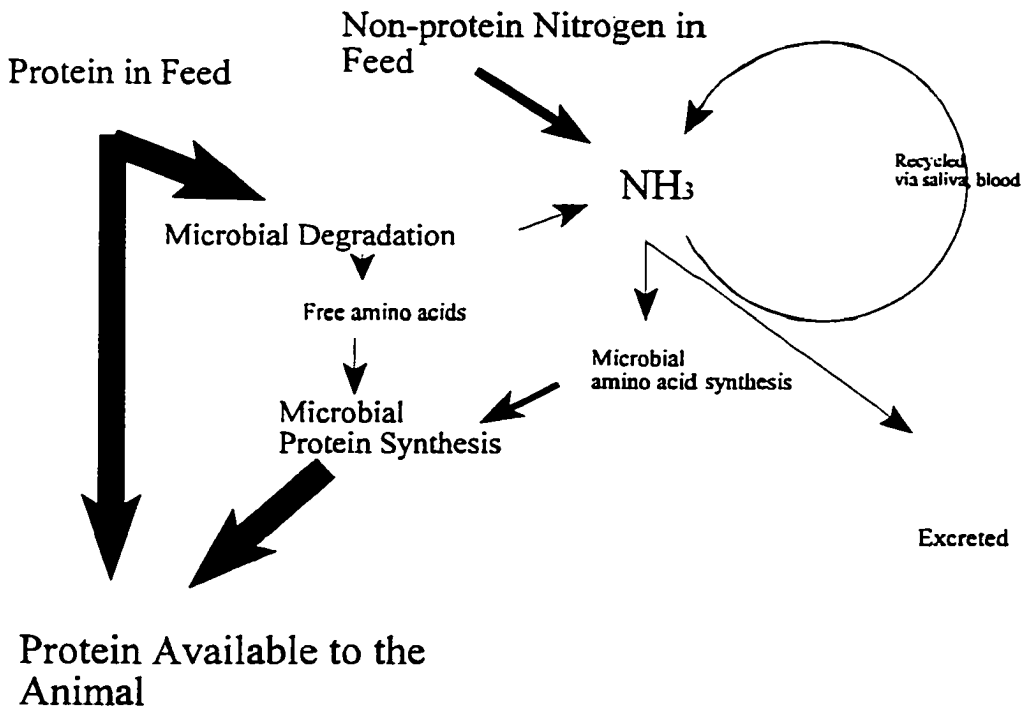


Figure 4a: Ruminant Digestive Pathway. A large portion of diet protein is used by microbes in the rumen. Thus a large portion of protein available to the animal comes mainly from microbes.

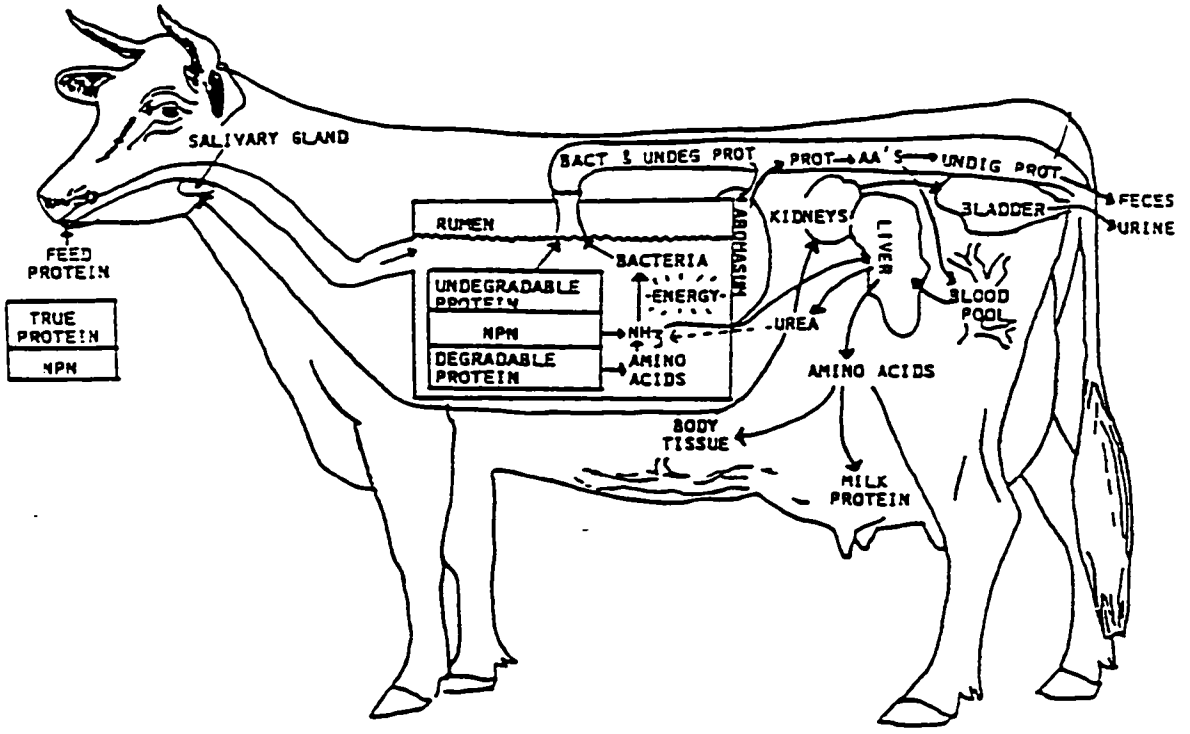


Figure 4b: Digestion of protein in the ruminant stomach.

In the abomasum (follows the rumen), the main enzyme pepsin, which is active at the abomasal pH 2, degrades the rest of the protein (Asplund, 1994). Further digestion in the small intestine is then achieved by proteolytic enzymes in liver secretions, pancreas, and in small intestinal mucosa (Church, 1988). Both essential and non-essential amino acids from dietary protein as well as microbial protein are absorbed into the portal vein and travel to the liver. In the liver, they join the body's general amino acid pool to be used for protein synthesis or pass directly into the systemic circulation to other tissues of the body. Not all of the amino acids are used for protein synthesis. Those in excess will be passed back into the liver where by the process of deamination, they can be converted to ammonia and keto acids.

1.2.2 The Availability of Essential Amino Acids

In dairy cows, most of the protein available for digestion by the animal comes not from the feed but from microorganisms in its rumen (Hungate, 1966). Microbial rumen degradation of feed proteins may exceed microbial protein synthesis and often results in a net loss of dietary protein, a situation which is particularly damaging to productive ruminants such as dairy cows which have high protein requirements.

Several options are being pursued to meet these protein demands. Studies have indicated that feeding proteins which are resistant to microbial breakdown in the rumen (or feeding proteins/amino acids postruminally) significantly increased production of milk and milk protein (Menneke *et al.*, 1991; King *et al.* , 1990; Oldham *et al.*, 1985; Orskov *et al.*, 1987; Faldet and Satter, 1991). In view of this, several groups have been trying to create "by-pass" protein sources that would resist degradation in the rumen (Beauregard *et*

al., 1995; McNiven *et al.*, 1994; Robinson *et al.*, unpublished).

There are two commonly used methods available for protecting proteins from degradation by rumen microorganisms; chemical and physical approaches. In the chemical approach, certain chemical agents such as formaldehydes or tannins form cross linkages with amino and amide groups which decrease the solubility of proteins at the pH of the rumen. Chemically-treated proteins are subsequently made available to the animal by destruction of these linkages in the acidic abomasum (Chalupa, 1975). In the physical approach, grains and forages are heat-treated so that the resulting product has decreased solubility and degradation in the rumen (Stern *et al.*, 1985).

Protection of proteins by some chemical and physical treatments has been shown to increase milk production in dairy cows (Robinson, unpublished; McNiven *et al.*, 1994). However, such techniques increase the cost of feeding and maintenance. They can also modify the protein rendering it undigestible in the intestine. These techniques can also selectively decrease availability of limiting amino acids like lysine (Clark *et al.*, 1992; Robinson *et al.*, 1995).

The impact of rumen microbial degradation is not limited to protein quantity but also affects protein quality. Ruminally synthesized microbial protein does not possess an ideal essential amino acids (EAA) balance and is insufficient to meet the demands of lactation in high producing cows (Chalupa, 1975). The result is a fairly large deficit in the amino acids methionine (M) and lysine (K), and a lesser deficit in other amino acids including threonine (T) and leucine (L) (Orskow *et al.*, 1987; Chalupa, 1975; Stern *et al.*, 1985; Ashes *et al.*, 1995). Thus, the effectiveness of protected feedstuffs will be limited

by their amino acid composition. In fact no diet, be it protected or not, currently provides the required essential amino acids profile to meet demands of high producing cows (Orskow *et al.*, 1987).

1.3 Creation of EAA Enriched Proteins

1.3.1 Early Attempts

An alternative approach would be to create an ideal protein that would be resistant to ruminal degradation. Previous attempts were based on the design of simple genes made of repeating segments coding for essential amino acid (Doel *et al.*, 1980; Jaynes *et al.*, 1985; Beauregard *et al.*, 1994). This approach presented several problems: (1) highly repetitive DNA segments were unstable; and (2) polypeptides with repeating sequences deviate from the usual, natural protein format and were not useful for nutrition (Boeble and Baker, 1982).

The approach used by our group was to design a *de novo* protein that has a high content of selected amino acids but would also resemble natural proteins in terms of its structural stability. A major emphasis was placed on encoding protein structural functions in the amino acid sequence using the new, emerging design principles made available in the late 1980's (Degrado, 1988). Using the consensus residue properties approach, a synthetic protein, Milk Bundle-1 (MB-1) enriched in four essential amino acids (methionine (M), threonine(T), lysine (K), and leucine (L)) was designed (Beauregard *et al.*, 1995).

1.3.2 MB-1: A Possible Solution

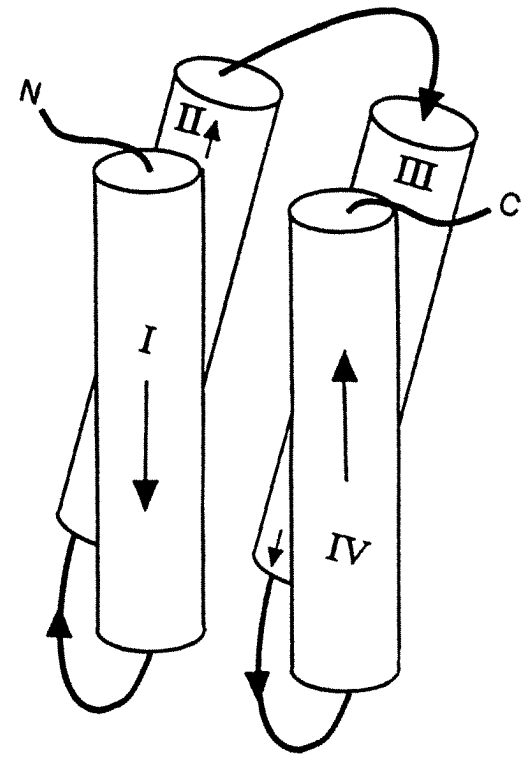
MB-1 is a *de novo* designed protein recently developed by Beauregard *et al.* (1995), with 57% of its amino acids being either K, M, T, and L. Emphasis was placed

on designing a protein that would fold into a compact structure, a departure from earlier attempts in this area (Jaynes *et al.*, 1985; Kangas *et al.*, 1982; Beauregard *et al.*, 1994; Boebel and Baker, 1982; Maldague *et al.*, 1991). MB-1 was designed to adopt the α-helical bundle fold and is engineered in a way to maximize its lifetime in bacteria. This approach has been used because MB-1 is intended to be expressed intracellularly. Thus, the protein would be contained within rumen microbes, and protected from degradation until it became available for digestion as the microbes reach the small intestine. Another possible approach would be transgenic feed crops, with the MB-1 gene inserted into the plants' DNA. This approach offers several advantages: it does not involve industrial processing of feedstuff, it is not likely to provide overprotection in the intestine because it does not involve a chemically resistant matrix, and it simply provides selected amino acids known to be limiting for animal performances.

1.3.3 Expected Structure of MB-1

The sequence of MB-1 was designed to adopt the α-helical bundle conformation (Figure 5) . Figure 5a shows the designed conformation of MB-1, four α-helices connected by loops, to form a bundle. The α-helical bundle fold is driven by collapse of the helices to form a hydrophobic core (Betz and DeGrado, 1995). Figure 5b shows the 100 amino acids used in MB-1 in the sequence that encodes for the bundle. The bundle is represented as opened up and flattened out, the dashed line circles represent amino acids intended to be in the hydrophobic positions.

A



B

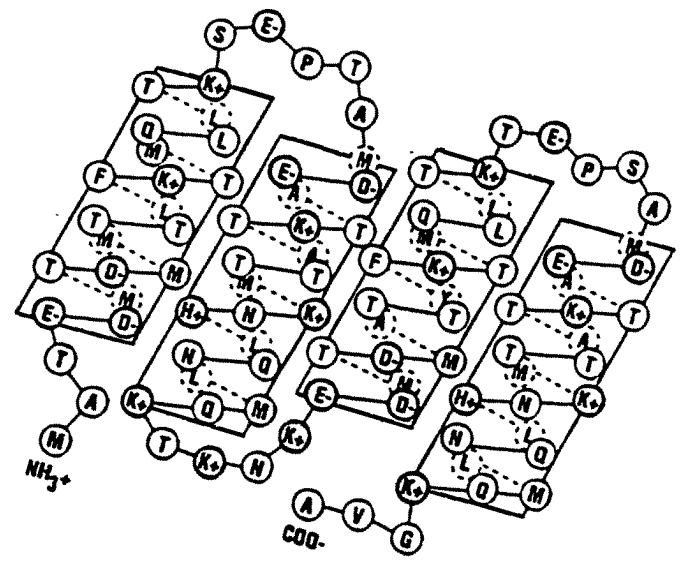


Figure 5: The designed fold for MB-1: α-helical bundle (a) cylinders represent helices that make up MB-1 (b) the external face of the MB-1 bundle after opening and flattening, dashed circles are the amino acids in hydrophobic positions

Fluorescent probes which have readily interpretable emission properties can be specifically inserted into proteins to reveal information on their structure and dynamics. In the design of MB-1, the fluorescent aromatic amino acid tyrosine was strategically designed to be in the hydrophobic core of the protein (Figure 6). Therefore information about folding of the protein can be inferred based on the spectral emissions of tyrosine (Stryer, 1968). For example, the addition of quenching agents will change the response of the spectral properties of tyrosine and indicate whether or not the fluorophore is exposed to the solvent (Stryer, 1968).

1.3.4 Tyrosine 62 as a Fluorescence Probe

As mentioned, aromatic amino acids display characteristic fluorescence spectra. In this study, these properties of fluoresence of Y62 were used for characterization of MB-1 structure. This section presents a review of the basic principles of fluorescence.

Molecules can absorb light energy which corresponds to the change in energy between two electronic energy levels. The atomic nuclei, which are held together by electron bonds, also play a role in determining which wavelengths are absorbed. The pull of the nuclei on the electrons will determine the spacing between excited and ground states. Therefore the characteristic energy of a transition, and the wavelength of radiation absorbed is a property of a group of atoms, not the electrons themselves. This group of atoms is known as a chromophore (Pavia *et al.*, 1979). When radiation is absorbed a chromophore may do several things: (1) produce heat, giving way to a rise in temperature; (2) change in chemistry, which puts energy into altered bonding structures; (3) become luminescent, which is the emission of a photon of the same or lower energy

(Tinoco *et al.*,1978).

There are two types of luminescence each characterized by the nature of its ground and excited states. One type, fluorescence, comes from the relaxation from the S_1 excited state to the ground state S_0, (see Figure 7). In this case, the electron in the higher energy orbital has the opposite spin orientation as the second electron in the lower orbital.

The molecule's electronic structure is changed by the absorption of light leading to an excited state. This can be measured as an absorption spectrum. The "excited" molecule loses some energy by non-radiative processes and emits light at a lower energy than was absorbed. This emission can be measured as a fluorescence spectrum. Thus the emission spectrum is shifted to a longer wavelength than the absorption spectrum (Stokes shift) (Lakowicz, 1983).

Substances which display fluorescence are known as fluorophores. There are two types of fluorophores used in fluorescence spectroscopy of proteins: (1) intrinsic, and (2) extrinsic. Intrinsic fluorophores are a part of the protein. The fluorescence of proteins originates from one or more of the three aromatic amino acids: phenylalanine, tyrosine and tryptophan. The position of the maximum of the fluorescence spectrum of tryptophan residues in proteins falls within the limits of 307 to 353 nm; from 303 to 306 nm for tyrosine (Figure 8) and 280 to 282 nm for phenylalanine. In MB-1 the intrinsic fluorescence comes from tyrosine in position 62.

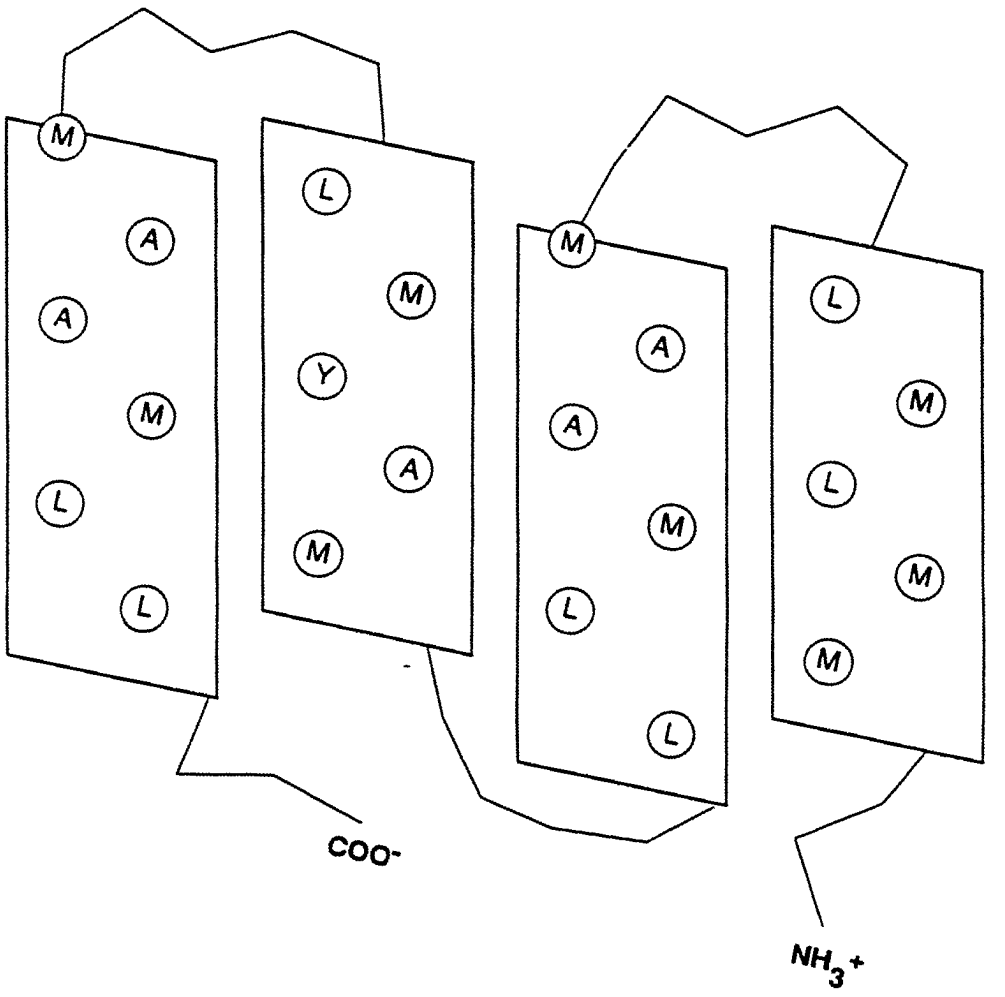


Figure 6: The internal face of the MB-1 bundle showing only the amino acids designed to make the core of the protein interior. Tyrosine 62 is represented by the letter code "Y".

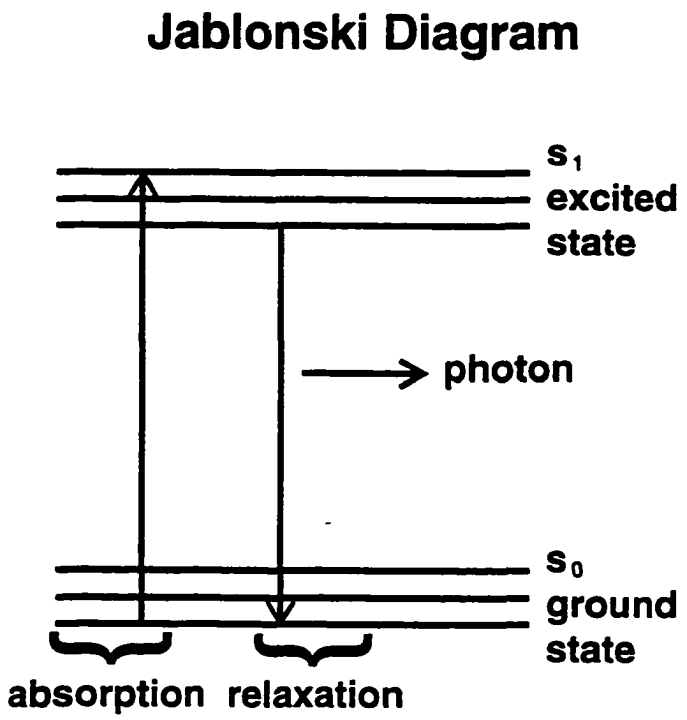


Figure 7: Jablonski diagram: Electrons absorb energy in the ground state and are excited to a higher energy level (S1). As they relax back down to the ground level (S0), the energy is emitted as a photon.

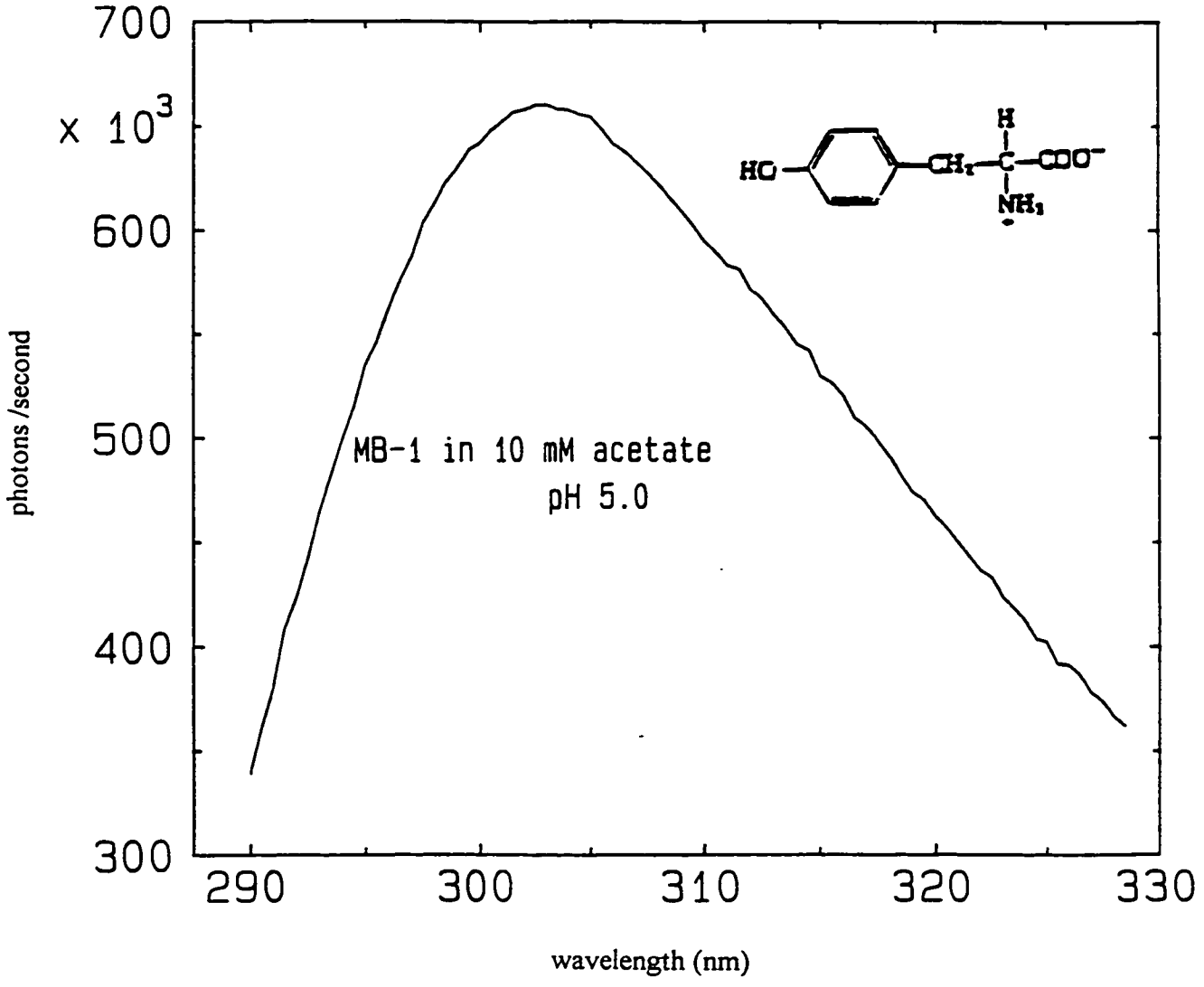


Figure 8: MB-1 fluorescence spectrum with maximum emission at 302-306nm. Inset: the structure of the aromatic amino acid tyrosine.

The fluorescence of these amino acids is widely used as an indicator of the conformation of the proteins (Lakowicz, 1983). This is because the fluorescence of the fluorophore is affected by the environment in which it is found. For example, if a protein is unfolded, the environment of its fluorophore is changed and therefore its emission spectrum may be changed. Addition of substances such as quenchers also change the environment of the fluorophore and can reveal information about the protein structure. An example of this will be given in section 3.1.

The second type of fluorophore used in this work is an extrinsic one. It is not a part of the protein, but rather another molecule that can be used as a probe that interacts with the protein and display fluorescence. For example, 1-anilino-8-napthalene-sulfonic acid (ANSA) binds to hydrophobic clusters and becomes fluorescent when bound. Otherwise it is non-fluorescent in aqueous solution (Figure 9).

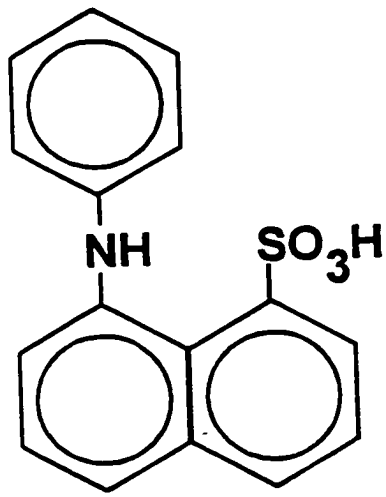


Figure 9: The structure of ANSA.

1.4 Prediction of MB-1 Stability in Rumen

A recent study conducted on MB-1 assessed its stability and degradability in a rumen- like environment . MB-1 was exposed to proteolytic degradation using conditions developed to mimic rumen degradation (MacCallum *et al.*, 1997; Omar, 1996). In addition to degradability, assessment of structural stability of MB-1 in the rumen environment was undertaken. It is recognized that compact folding of proteins confers a resistance to degradation by proteases (MacCallum *et al*, 1997; Arnold and Zhang, 1994; Goldberg *et al.*, 1978; Goldberg and Goff, 1986; Parsell and Sauer, 1989; Liao, 1993). Thus, we wanted to assess the impact of the rumen environment on folding stability and its consequent contribution to proteolytic degradability. MB-1 was also exposed to a range of temperatures to unfold the protein using heat, in a way to monitor its thermal stability.

1.4.1 Digestibility of MB-1

Degradability studies mentioned above (MacCallum *et al*, 1997) predict that MB-1 will be rapidly (<20min) and totally degraded if exposed to the rumen environment. This property will be a disadvantage if MB-1 leaks (from the cell) or is deliberately secreted into the rumen. However, when delivered to the abomasum, MB-1 degradability will become an advantage. Physical and chemical treatments for protein protection can lead to overprotection, while some non-protected feeds are not fully digestible. The very nature of MB-1 and results from this study, suggest that MB-1 will be fully degraded in the intestine.

In a previous study (MacCallum *et al*, 1997), two natural proteins of a similar size to MB-1 were used for comparison. These RNase A, cyt *c* are well known for their

folding stability and their resistance to proteolytic degradation (Privalov, 1979; Imoto *et al.*, 1974). As predicted, they were not as degraded as MB-1. One explanation for this result is that cyt *c* and RNAse A both contain disulfide bridges whereas MB-1 has none. It has been recognized that these structural features provide protection against degradation in the rumen (Mahadevan *et al.*, 1980; Spencer *et al.*, 1988).

A number of studies on protein degradability in rumen fluids have focused on the analysis of isolated proteins. Among the proteins studied, caseins and other milk proteins have been shown to degrade in few minutes (Spencer *et al.*, 1988; Hancock *et al.* 1994). Thus, they are similar to the predicted lifetime of MB-1 in the rumen. Similar results were obtained for pea proteins like convicilin, vicilin as well as for other proteins like those of rapeseed and lupin (Spencer *et al.*, 1988). Therefore, MB-1 degradability in the rumen would compare to that of a number of proteins found in the diets of ruminants. Other proteins such as bovine serum albumin, RNAse, and plant albumins have been shown to be more resistant to rumen degradation than MB-1. These have been shown to resist degradation for about 8 hr (Mahadevan *et al.*, 1980; Spencer *et al.*, 1988; Hancock *et al.*, 1994).

1.4.2 Thermal Stability of MB-1

To further explore the reason for the rapid degradation of MB-1 in rumen conditions, MB-1 folding stability was investigated. Several studies have demonstrated that unfolding promotes proteolytic degradation as it leads to exposure of target groups (Goldberg *et al.*, 1978; Liao, 1993; Schein, 1989; Yang and Tsou, 1995; Huang *et al.*, 1994). If MB-1 was to become unfolded at rumen temperature (39 °C), then it would be

highly susceptible to rumen degradation. Thermal denaturation studies were also done on MB-1 in a solution with pH, salt and volatile fatty acid content similar in nature to rumen levels. Modification of the tertiary structure was reflected by changes in the fluorescence properties of the aromatic residue, tyrosine 62. A melting temperature (Tm) of 39.1 +/- 1.7 °C was obtained for MB-1 (MacCallum *et al*, 1997).

This result indicates that 50% of MB-1 population has an unfolded tertiary structure at 39°C, which corresponds to the temperature of the rumen. The denaturation (transition region) of MB-1 occurs over a broad range of temperature as compared to RNAse A and cyt *c*. This suggests less cooperativity in thermal unfolding for MB-1, a feature of other *de novo* designed proteins for which temperature (Fezoui *et al.*, 1995; Kuroda, 1995) or chemical denaturant (Hecht *et al.*, 1990) were used for denaturation studies.

1.5 Importance of Protein Stability in its Digestibility:

Presently, there is considerable interest in gaining a better understanding of the forces that contribute to the stability of globular proteins. Stability has long been a concern for *de novo* synthesized proteins. With a better understanding of the principles governing stability, better *de novo* proteins can be designed. Of all the proteins under investigation to date, MB-1 has been shown to be the best synthetic protein ever to be designed for nutrition. It is expressed *in vivo*, is folded and has several features of native (natural) proteins. Its resistance to proteases is rather weak but compares to that of some natural plant proteins. However, the labile nature of MB-1 has been identified as a potential problem that would limit both expression levels in plants, and resistance to

degradation in cow rumen. The folding stability of MB-1 must be addressed. Low thermal stability leads to increased proteolysis (Liao, 1993; Parsells, 1989; Goldberg, 1978; Arnold and Zhang, 1994). Considering that proteolytic degradation may occur in the organism where it is expressed, it is believed that MB-1 stability must be improved in order to make it appealing to Canadian plant biotechnology corporations as a viable candidate for expression in feed crops. By finding out the contributing factors responsible for low thermal stability, these parameters can be improved upon with mutations in an attempt to increase proteolytic stability. The ultimate goal is to create high quality proteins with properties that will appeal to potential industrial partners and lead to creation of a transgenic feed crop with high nutritional value.

1.6 Thesis Objectives:

MB-1 was designed to adopt a specific tertiary fold, the α-helical bundle. In order for MB-1 to be useful, its tertiary structure and behaviour in solution must be investigated. For improvements tobe made to the design, the tertiary structure must be known. Once the structure and behaviour of the protein in solution are known, mutations can be introduced which will increase the conformational stability in solution and in rumen conditions. Principles for increasing stability are known, but the exact conformation of MB-1 is not. Therefore, the characterization of MB-1 would provide new and useful information concerning its *in vivo* function.

The main objectives of this study are:

- To probe hydrophobic core formation using tyrosine spectral emission.
- To determine the compactness of the protein bundle.

- To compare the behaviour of the hydrophobic core of MB-1 to that of natural proteins.
- To assess the folding of MB-1.
- To suggest further mutations that would increase the stability of MB-1.

2. General Materials and Methods

2.1 MB-1 Production Protocol

An overnight culture of the *Escherichia coli* TB-1 strain containing the MB-1 gene integrated in the pMal-c2 expression vector (New England Biolabs, Mississauga, ON) was diluted 1/100 in 1 litre of Luria Broth supplemented with 100ug/ml ampicillin. The culture was grown for 2.5h at 32°C with agitation (A_{600} = 0.2 to 0.4), IPTG was added to a final concentration of 0.5 mM and incubation continued for 3.5h (A_{600} = 0.6 to 0.8).

Cells were harvested by centrifugation (4300rpm, 1 h) and resupended in buffer A (10 mM Tris, 200 mM NaCl, 10mM EDTA and 1 mM NaN_3, pH 7.4). Cells were lysed by sonication in an ice/methanol bath to maintain a low temperature (10 pulses of 30 s with a pause for cooling between each pulse), and NaCl was added to a final concentration of 700 mM in order to precipitate cell debris for removal by centrifugation. The resulting bacterial extracts contained a fusion of maltose binding protein with milk bundle protein (MBP-MB1). The supernatant was then loaded onto an amylose column for affinity chromatography.

Twenty mL of amylose resin (New England Biolabs, Mississauga, ON) was poured into a column and washed with 10 volumes of buffer A. The column was then attached to a solvent delivery system at 1 mL/min. The bacterial extract containing the fusion protein was then loaded onto the column using a sample loop. The fusion protein was eluted by applying an elution buffer (buffer A + 10 mM maltose, pH 7.4). The absorbance of the

eluted sample was recorded at 280nm. The fractions corresponding to the eluted peak on the chromatogram were pooled in a dialysis bag (Spectra/Por, Houston, TX; MWCO 3,500 Da) for cleavage.

Cleavage of the fusion protein was achieved by adding 50 μL of 1 mg/mL factor Xa (New England Biolabs, Mississauga, ON) to the pooled sample inside a dialysis bag. The sample was then dialysed using 3.5 kDa cut off dialysis bags (Spectra/Por, Houston, TX; MWCO 3,500 Da). Cleavage was conducted in 20 mM Tris (pH 8.0), 100 mM NaCl, and 2 mM $CaCl_2$ at 4 °C overnight. The bag was then transferred to 2 L of buffer B (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and dialyzed for 2 hr to remove the maltose (according to the protocol described in New England Biolabs). Separation of MB-1, MBP and factor Xa was achieved by ion-exchange chromatography. The sample was loaded onto a diethylaminoethyl sepharose (DEAE pH 8.0) fast flow column (Pharmacia, Baie D'Urfé, Québec) and eluted by washing the column with 100 volumes of buffer B. Four mL fractions were collected and assayed for protein content using a bicinchonic acid protein detection kit (Sigma, Oakville, ON).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using tricine buffers was conducted in a Miniprotean Bio-Rad apparatus (Beverly, MA). Acrylamide gels made of 12% resolving gel and a 3% stacking gel (Sigma Chemical Co., St.Louis, MO) were prepared. Electrophoresis was conducted in Tris-tricine buffer (0.1 M Tris pH 8.25, 0.1 M Tricine, 0.1% SDS) for 1 hr at 100 V and 30 mA. Tricine was used to ensure optimal band resolution of small molecular weight proteins (Schagger and Von Jager, 1987). Proteins were stained using the Bio-Rad Silver staining kit (kit # 1610443)

according to manufacturers instructions (Bio-Rad, Mississauga, ON). Samples of the fusion protein as well as the after cleavage product were loaded on the gel to ensure complete cleavage had occured. Purity of MB-1 samples after ion-exchange chromatography was based on absence of bands of a different molecular weight than MB-1 (Figure 10). Figure 10a shows the SDS-PAGE analysis of various stages of preparation at various stages: lane 1, molecular weight standards; lane 2, 3, the cells before and after induction with IPTG, respectively, lane 4, cell lysate after fusion protein has been released by sonication; lane 5, cellular debris removed by centrifugation; lane 5 fusion protein (from amylose column) before cleavage with factor Xa; lane 7 after cleavage with factor Xa, MB-1 is now separated from the MBP.

Figure 10b shows the SDS-PAGE analysis of MB-1 purification after the DEAE sepharose column: lane 1, molecular weight standards;lanes 2-7, protein composition of MB-1 loaded in various amounts. Lanes 4 and 7 containe more protein than lanes 2, 3, 5, and 6. Purity of MB-1shown is greater than 95%.

Fractions identified as pure protein samples on SDS-PAGE were pooled and concentrated by ultrafiltration (Amicon, Oakville, ON) using a membrane with a molecular weight cut off of 3 kilodaltons. Samples were concentrated under nitrogen to a volume of 3-5 ml and then further concentrated to a smaller volume using Centricon 3 concentrating tubes 3, 000 MWCO (Amicon, Oakville, ON).

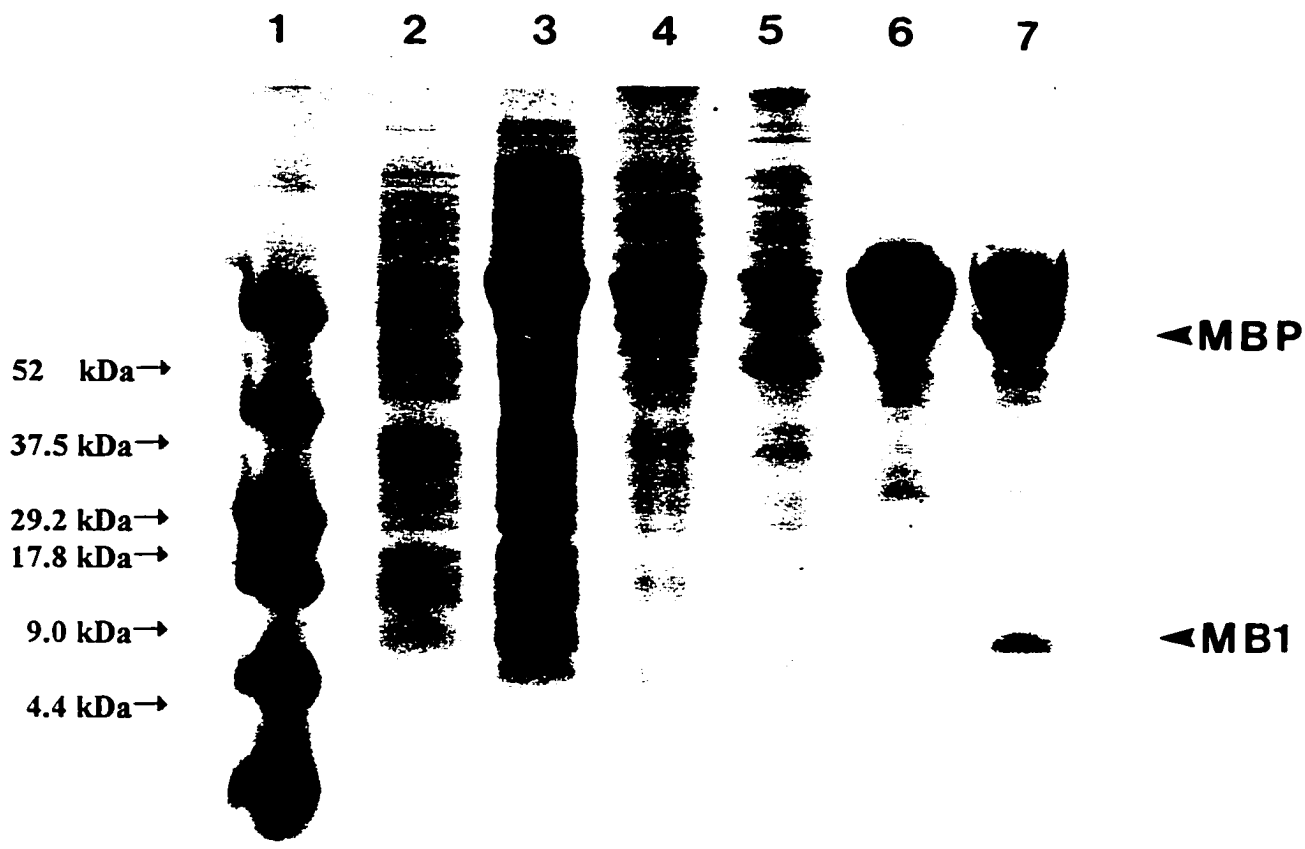
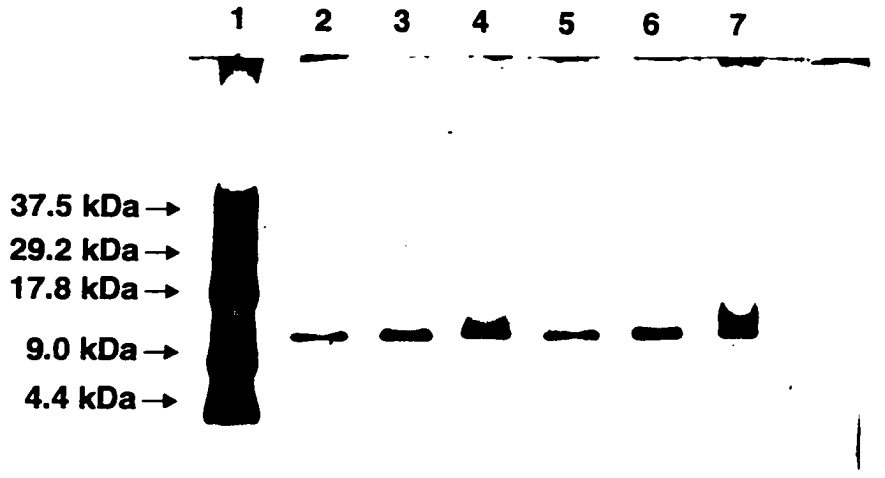


Figure 10a: SDS-PAGE analysis of MB-1 purification: Lane 1 contains molecular weight standards, lanes 2, 3, cells before and after induction with IPTG respectively; lane 4, cell lysate after sonication to release the protein, and lane 5 shows the cellular debris removed with centrifugation; lane 6, fusion protein (MB-1-MBP) before cleavage with factor Xa; lane 7, fusion protein after cleavage, MB-1 is now separated from MBP.



10b: SDS-PAGE analysis of MB-1 purification off the DEAE sepharose column: lane 1, molecular weight standards; lanes 2-7, protein composition of MB-1. Lanes 4 and 7 contain 100 fold more protein showing that the protein is pure at >95% (protein composition).

2.1.1 Quantitation of MB-1

The concentration of MB-1 in the sample was determined by using the bicinchoninic acid assay (Sigma, Oakville, ON) along with standards of known protein quantity (BSA 1mg/mL supplied with the BCA kit, Sigma.) Absorbance at 562nm was measured by a spectrophotometer and compared with the absorbance of known standards. Average yield for one preparation was 5 mg.

2.1.2 Sample Preparation for Experiments

All experiments presented in this thesis were performed in 10mM acetate buffer pH 5.0. Samples were dialysed in 3.5 kDa molecular weight cut off dialysis bags against 200 volumes of the appropriate buffer at 4 °C overnight. The following day, protein concentration was measured again to confirm that it had not changed during dialysis.

2.2 Fluorescence Experimental Set Up

Figure 11 illustrates the instrument set up of the fluorometer used for all fluorescence measurements presented here. The light source, a 75 watt xenon lamp, was used because of its high intensity at all wavelengths ranging upward from 250nm. The fluorometer is also equipped with two monochromators to select the emission and excitation wavelengths. Both monochromators are motorized to allow for automatic scanning of wavelength. These settings are controlled via computer software. The fluorescence is detected by photomultiplier tubes and quantified by the software.

The sample chamber is usually central to all components of a fluorometer. Light from the lamp enters the sample chamber through shutters. Light enters the cell containing

the sample and the photons emitted then enter the photomultiplier tubes. The signal is fed into the computer and is recorded as a graphical representation of photons emitted per second.

An emission spectrum is the wavelength distribution of the emission measured with a single constant excitation wavelength. The usual units of wavelength are nanometers (nm). The recorded emission spectrum represent the photon flux emitted at each wavelength, over a pre-determined wavelength interval, determined by slit widths and dispersion of the emission monochromator. A band pass of 4nm/mm was used. The wavelength-dependant efficiency of the monochromators and of the photomutiplier tubes are corrected using correction files that were prepared by the manufacturer.

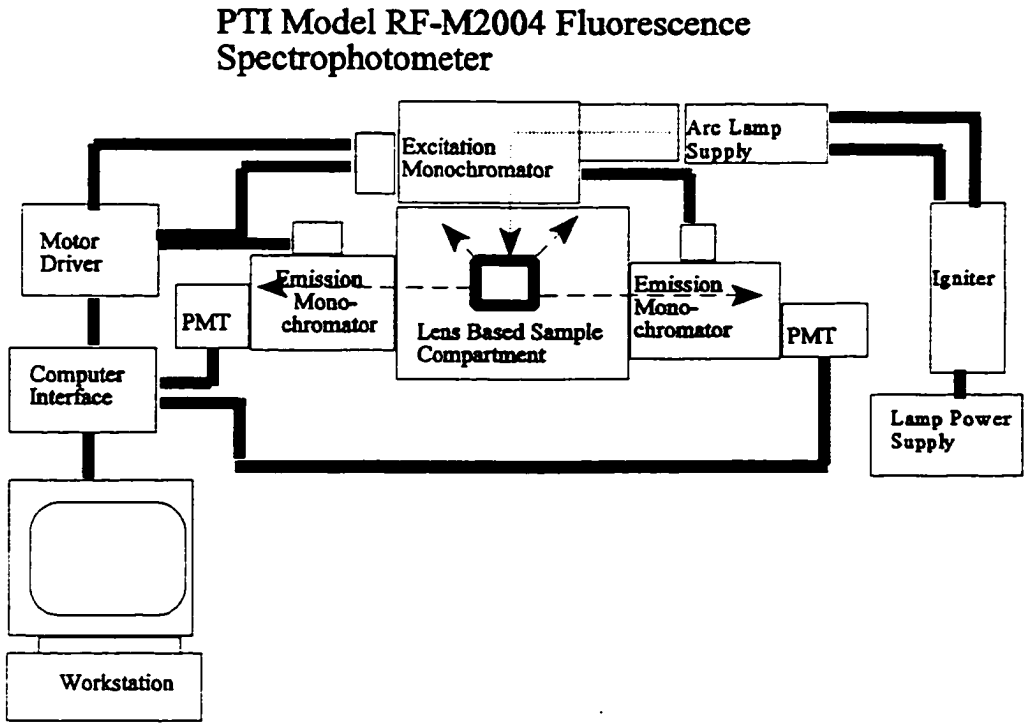


Figure 11: Experimental set up for fluorescence spectroscopy. Dashed line represents light path. Excitation wavelength is selected by the excitation monochromator, sample absorbs light and emits fluorescence in all directions. Fluorescence is recorded at a right angle and emission monochromators are used to scan the appropriate wavelength.

3. Fluorescence Quenching: Determination of Tyrosine Environment

3.1 Introduction

The first step in MB-1 characterization was to determine if the four helices of the MB-1 fold had come together through hydrophobic interactions to form a hydrophobic core as per design. Failure to achieve such a core would result in higher proteolytic degradation of MB-1. Fluorescence quenching was chosen for MB-1 characterization because of the strategically placed intrinsic chromophore, tyrosine (Y62), which was designed to be in the hydrophobic core of the protein. Since the environment of the side chain of tyrosine was designed to be in the non-polar core, therefore information about folding of the protein can be inferred based on the spectral emission of tyrosine. Addition of quenching agents will change the response of the spectral properties of tyrosine and indicate whether or not the fluorophore is exposed to the solvent. (Stryer, 1968).

Fluorescence quenching refers to any process which causes a decrease in fluorescence intensity of a given substance (Figure 12). For quenching to occur, the quencher and the fluorophore must come in contact with one another at some point. This important requirement results in the numerous applications of quenching experiments. For example, quenching can reveal how accessibile the fluorophores are to quenchers. If the protein is impermeable to the quencher, and the fluorophore is in the interior of the protein, then no quenching is expected. Therefore, quenching can reveal the location of a fluorophore in a protein (Lakowicz, 1983).

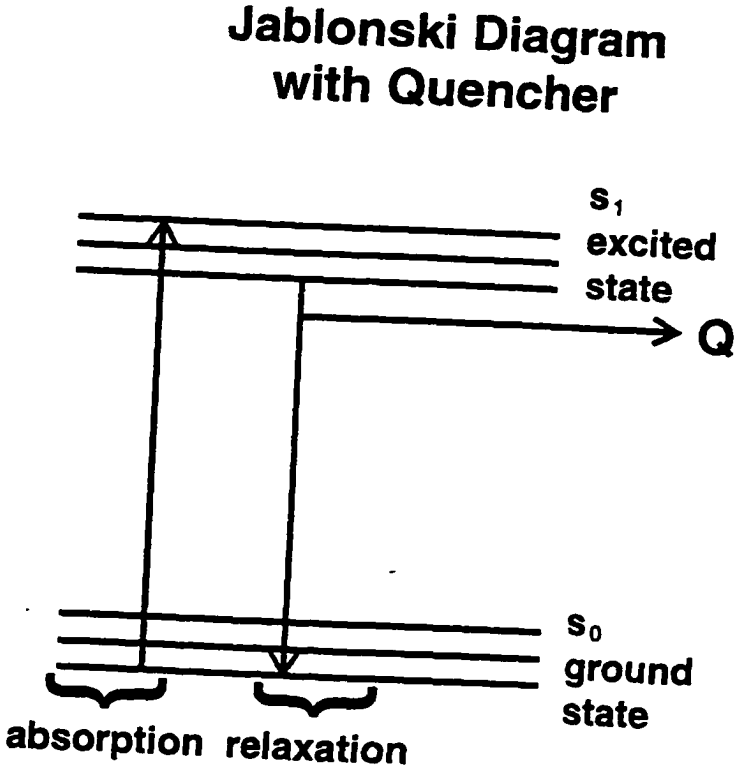


Figure 12: Jablonski diagram for a quenched system. Molecules in the ground state absorb energy in the ground state and are excited to a higher electronic energy level (S_1). The quencher then de-excites the molecule decreasing the number of photons emitted.

3.1.1 Static and Dynamic Quenching

There are two types of quenching: (1) collisional, (or dynamic), and (2) static quenching. Both static and dynamic quenching require molecular contact between the fluorophore and the quencher. In the case of dynamic quenching , the quencher must diffuse to the fluorophore during the lifetime of the excited state. Upon contact with the quencher, the fluorophore will return to the ground state without the emission of a photon. In the case of static quenching, a complex is formed between the fluorophore and the quencher, and this complex becomes non-fluorescent. In either event, the fluorophore and quencher must come into contact with each other.

3.1.2 Quenchers of Fluorescence

A wide variety of substances may act as quenchers. Quenching by these substances involves the transfer of energy from the fluorophore to the quencher allowing the fluorescing molecule to return to its ground state. Different quenchers quench different fluorescing molecules in different manners. Molecular oxygen, one of the best collisional quenchers, for example, quenches almost all known fluororphores. Aromatic and aliphatic amines are efficient quenchers of most unsubstituted aromatic hydrocarbons. Other collisional quenchers include citrate, xenon, hydrogen peroxide, acrylamide, bromide, and iodide (Lakowicz, 1983). Acrylamide is known to quench almost all fluorophores because of its ability to diffuse through a protein matrix. It is considered as highly effective as a quencher (Efftink and Ghiron, 1981). Citrate on the other hand cannot penetrate the matrix of a protein and will be efficient as quenching only if the fluorophore is exposed (Eftink and Ghiron, 1981).

Quenching studies are normally performed at a number of different concentrations of quencher in order to measure the rate at which the agent can diffuse to the site of the fluorescing residue (Liu, 1989). Interpretation of fluoresence quenching measurements is easier when a suitable model is used for comparison (Liu, 1989). In this study fully exposed L-tyrosine free in solution will be used as a model for comparison.

3.1.3 Objectives

Quenching experiments were performed using a negatively charged quencher, citrate, and a neutral quencher, acrylamide. These experiments are performed on MB-1 in its folded state, (with tyrosine presumably inside), and in an unfolded, denatured state (with tyrosine presumably exposed) . Comparison of MB-1 in the two states will be made using Stern-Volmer analysis. The aim of this study was to confirm the position of tyrosine 62 in MB-1's hydrophobic core.

3.2 Materials and Methods

Citrate quenching studies were performed with MB-1 in the native state, MB-1 denatured using 4M urea (with Y62 exposed), and with tyrosine free in solution. Samples denatured in urea were left to unfold overnight at room temperature Protein was used at a concentration of 0.2mg/mL in a 10mM acetate buffer, pH 5.0. Quenching was achieved by addition of small aliquots of citrate (0-0.25M) prepared in the same buffer. After the quencher was added the sample was mixed by pipette action and placed in the thermally controlled sample chamber. The sample was allowed to equilibrate for 5 min. The sample was excited at 280nm while emission spectra were recorded from 285 to 340nm. Fluorescence intensity at 302nm was recorded and used for calculations. All

measurements were made at 18 °C.

Control experiments were conducted using blanks to correct for buffer effects on the fluorescence. Three blanks were measured containing (1) 10mM acetate alone, (2) 10mM acetate + NaCl, and (3) 10mM acetate, NaCl, and citrate (at all concentrations used). NaCl was added to the blanks to maintain ionic strength, since citrate is a charged quencher. Concentration of NaCl varied from 0.41M to 0M as citrate varied from 0M to .25M. A stock solution of citrate (2.5M) was used at a working pH of 5.0.

Acrylamide quenching was also performed at a protein concentration of 0.2 mg/mL and acrylamide concentration varying from 0-0.6M. The appropriate blanks were subtracted from the experimental data obtained at each measurement. Three sets of experiments were performed in triplicate: (1) tyrosine free in solution, (2) MB-1 folded (tyrosine protected), (3) MB-1 unfolded by urea (tyrosine exposed).

The impact of a quencher on tyrosine fluorescence steady state emmission can be further described by using the following equation:

$$\tau = \quad 1 / k_f + \Sigma k_{nr} \qquad (1)$$

The fluorescence lifetime (τ), is proportional to the rate constant for fluorescence emission (k_f), but inversely related to the sum of all rate constant resulting in depletion of the excited state; i.e. $k_f + k_{nr}$, where k_{nr} (rate of decay for all non-radiative processes) includes k_q [Q]. k_q is the rate constant for quenching, while [Q] is the concentration of a given quencher. We see that depending on quenching (k_q) and the concentration of the quencher, we can modify τ: an increase in k_q [Q] results in an increase on the denominator side of equation 1, which results in a decrease in fluorescence lifetime (τ).

Such phenomena can be observed by steady state emission which is expressed as

$$I = \int_0^\infty I(t)\, dt \qquad (2)$$

where I = steady state fluorescence intensity, and I (t) = fluorescence intensity at time = t. I(t) is dependant on τ as shown by equation (3)

$$I(t) = I_0 e^{-t/\tau} \qquad (3)$$

Since τ is changed by quenching, we see that steady state fluorescence intensity will decrease when quenching occurs.

The Stern-Volmer equation describes the theory of dynamic quenching:

$$F_0/F_1 = 1 + K_D [Q] \qquad (4)$$

where F_0 = fluorescence intensity in absence of quencher

F_1 = fluorescence intensity with quencher

[Q] = concentration of quencher

K_D = Stern-Volmer quenching constant

The plot of F_0/F_1 yields an intercept of 1 on the y-axis and a slope equal to K_D. A linear Stern-Volmer plot is an indication of a single class of fluorophores, all of which are accessible to the quencher. Stern-Volmer plots will deviate from linearity when two populations of fluorophores are present and one is not accessible to the quencher

(Lakowicz, 1983). Comparison of slope (K_D) values will tell us if tyrosine is buried in the protein or located peripherally. A low slope value indicates protection from quenching, while a high slope value indicates the fluorophore is highly quenched. In many instances, the fluorophore can be quenched both statically and dynamically with the same quencher. The characteristic feature of static quenching is a deviation from linearity in the Stern-Volmer plot, particularly an upward curvature (Figure 13).

3.3 Results

Figure 14 shows the results of the citrate quenching experiment. An attempt to localise the tyrosine by measuring its fluorescence in the presence of a hydrophilic quencher was made. The Stern-Volmer analysis of all three systems (tyrosine alone, MB-1 folded, and MB-1 unfolded) is shown. Tyrosine free in solution (open circles) shows a high degree of quenching as indicated by the slope of the curve. The citrate not only collided with the exposed tyrosines but also remained in close proximity to statically quench the system. This indicated by the upward curvature in the plot. MB-1 was then exposed to citrate (open squares) and was found to be much less sensitive to quenching than the tyrosine alone. The absence of the upward curvature when MB-1 is folded suggests that the citrate was not in permanent close proximity to the lone tyrosine in MB-1, and the tyrosine was barely dynamically quenched even at a concentration of 0.25M citrate. In the third system, when MB-1 was unfolded exposing the tyrosine to solution, we see the return of the upward curvature indicating that the quencher was in close proximity to the tyrosine. These results indicate that when folded, MB-1 protects tyrosine 62 from citrate, in the hydrophobic core, as predicted by the designed structure of

MB-1.

Results of the acrylamide studies are shown in figure 15. Acrylamide quenches both the unfolded (open circles) and the folded (closed squares) forms of MB-1 as indicated by the upward curvature seen in both plots (folded and unfolded MB-1). This was expected since acrylamide is known to penetrate protein structures (Lakowicz, 1983). This experiment served as a control for the quenching of MB-1. It shows that the change in fluorescence seen with citrate upon MB-1 unfolding is due to quenching by citrate and not due to another fluorescent modulating process.

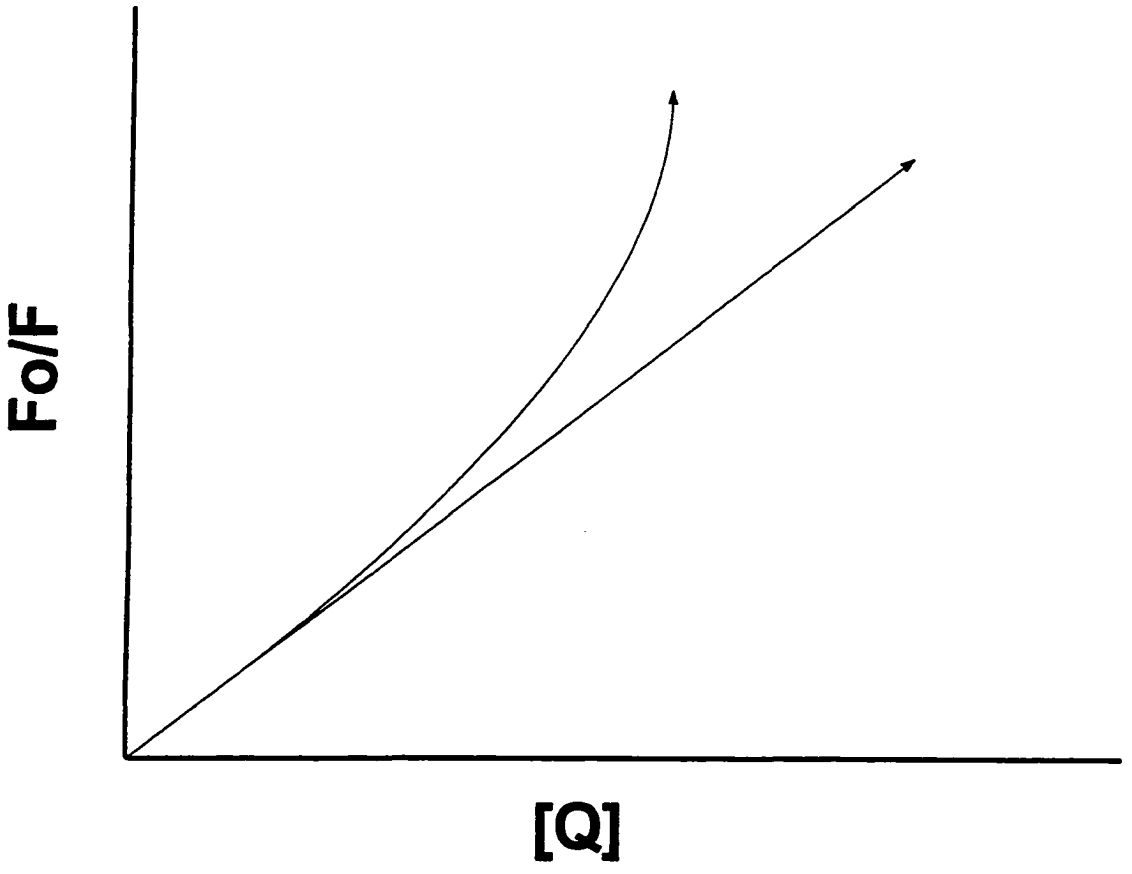


Figure 13: Typical Stern-Volmer plots. The upward curvature indicates increased static quenching; other line shows typical dynamic quenching. Fo/F represent the ratio of fluoresence with and without quencher.

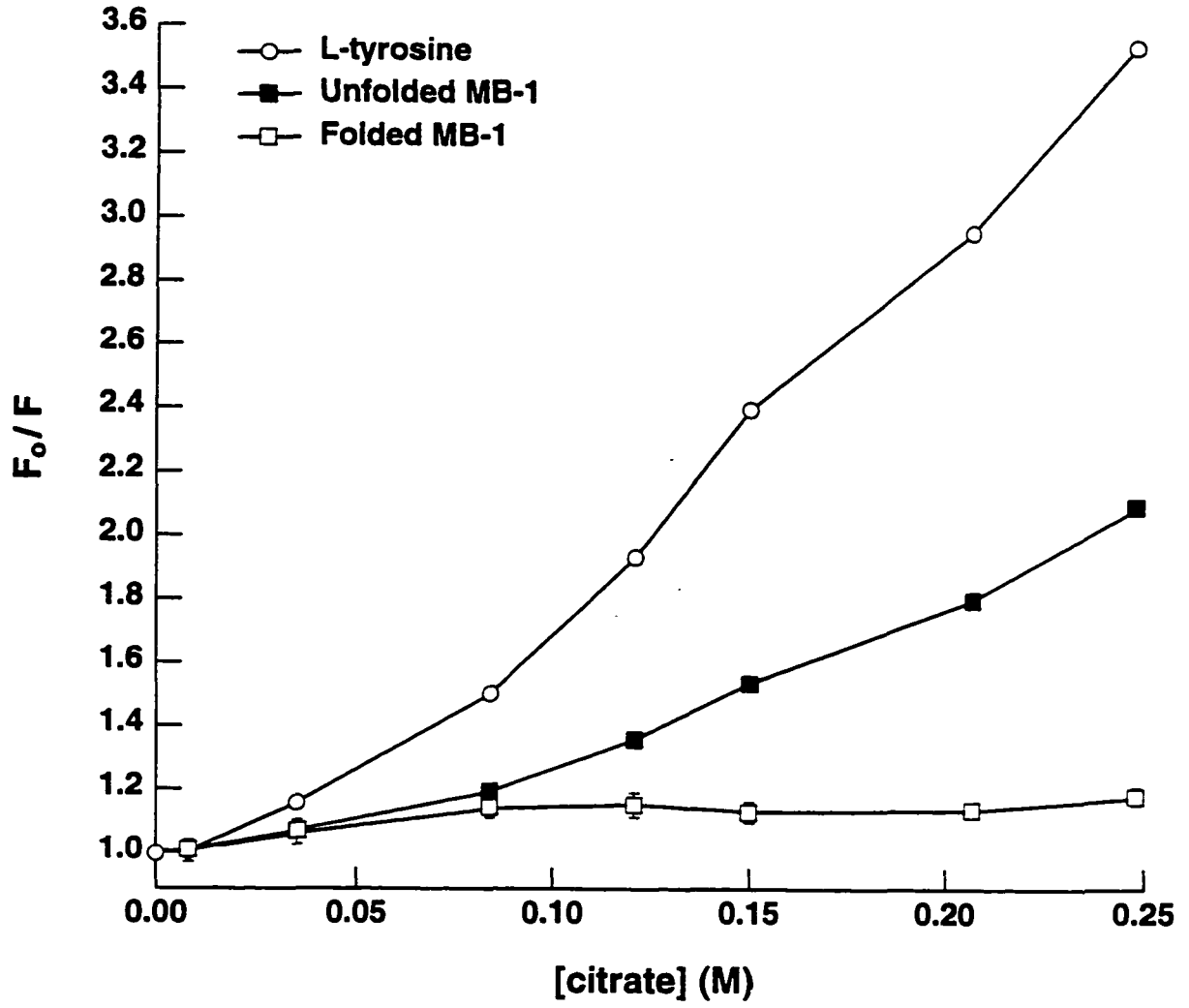


Figure 14: Stern Volmer analysis of citrate quenching on native and unfolded MB-1. Effects of urea on Y62 fluorescence were corrected as described in methods.

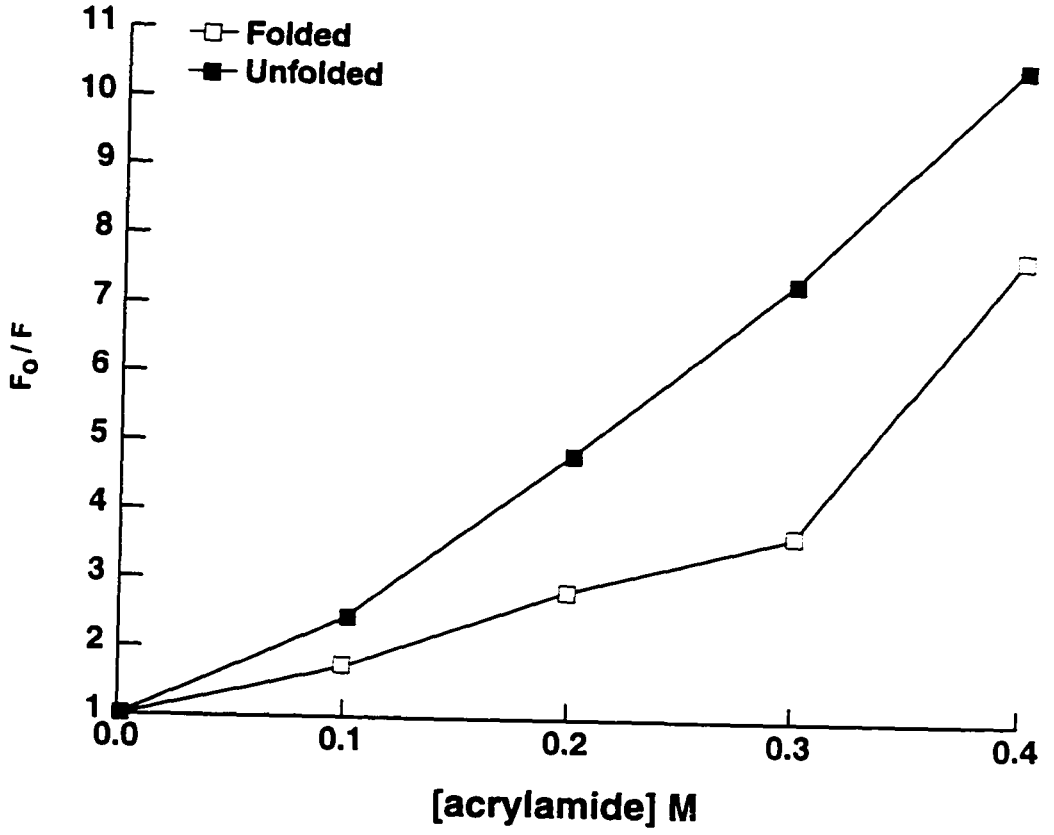


Figure 15: Stern Volmer analysis of acrylamide quenching experiments on MB-1 in the native and unfolded states. Effects of urea on Y62 fluorescence were corrected as described in methods.

3.4 Discussion

These quenching experiments are often used to indicate the placement of an aromatic residue (Pittman, 1995; Kuwajima, 1990; Liu, 1989). Kasprazak and Weber (1982) used citrate quenching to distinguish between tyrosines in the core and exposed tyrosines on the protein bovine pancreatic trypsin inhibitor. They found that citrate was the most effective quencher of tyrosine. Liu (1989) used citrate as a quencher to determine the position of the lone tyrosine (believed to be in the hydrophobic core) in the lima bean trypsin/chymotrypsin inhibitor. Liu found that by using a charged quencher such as citrate, the quencher could not access the fluorophore as it was somehow protected, presumably in the hydrophobic core. When the protein was unfolded by breaking the disulphide bonds, the tyrosine was quenched by citrate, indicating that it was indeed protected by the hydrophobic core formation. Leavis and Lehrer (1978) concluded that citrate quenching of tyrosine has both dynamic and static components.

Eftink and Ghiron (1976) used acrylamide to quench the buried tryptophan in ribonuclease T1, believing that it would be protected. Unexpectedly, they found that acrylamide could diffuse to the core and penetrate the folded protein to collisionally quench the fluorophore in the fully folded protein. Acrylamide is now commonly used to measure the accessibility of aromatics in the protein matrix (Eftink 1981). Acrylamide can diffuse through structural fluctuations in the protein which occur on a nanosecond timescale.

Other *de novo* design projects have also applied this method of characterization. Lee *et al.*, (1997), used acrylamide quenching to determine the location of tryptophan in

their four helix bundle. In another successful design project, by the name of "Felix", Hecht *et al.* (1990), also used intrinsic fluorescence to characterize the four helix bundle. This technique has been applied successfully to MB-1. Citrate and acrylamide quenching experiments both indicate that tyrosine (62) is located in the hydrophobic core of MB-1 as per design. Tyrosine 62 was expected to be in position "d" of helix III (Figure 16a, 16b, see also Figure 6), and our results support this prediction. Our results also show MB-1 has a normal hydrophobic core, which is disrupted by urea, and penetrable by acrylamide, two properties of natural proteins.

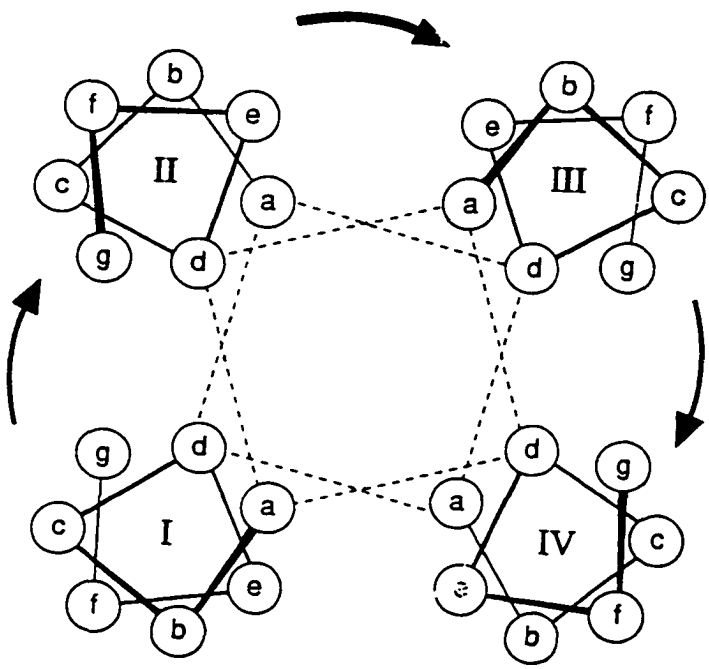


Figure 16a: MB-1 Structure showing the position of Tyrosine 62. Top down view of the four helices of MB-1 showing the heptad positions, a,b,c,d,e,f,g,h. Tyrosine was designed to be in position "d" of helix III, at the center of the helix (second turn).

Figure 16b: Computer model of the predicted tertiary structure of MB-1. The position of tyrosine is indicated by an arrow, in the protein core.

4. ANSA Binding Studies: Compactness of the core of MB-1

4.1 Introduction

In the last few years, considerable progress has been made in understanding what constitutes a protein fold. However, most designed α-helical bundles often adopt a fluctuating structure, with hydrophobic cores less well packed than native proteins (Kyte, 1995). This loosely folded structure has been termed molten globule state.

The molten globular state (MGS) of proteins is defined by the presence of definite secondary structural elements, with fluid tertiary structure (Ptitsyn, 1994). Various physical measurements have been made on proteins characterized as MGS. Circular dichroism studies have shown that they retain α-helical and β strand structures. It is thought that the MGS represents the random coil that has collapsed to a globular state due to the hydrophobic effect (Kyte, 1995). The protein will remain fluid in solution with a constantly changing conformation; rather than having one stable native structure, i.e. it will have a variety of conformations with similar energy (Kyte, 1995). Some proteins show molten globular states in their unfolding pathways (Shi *et al.*, 1994; Itzhaki, 1994). Natural protein folding intermediates believed to be MGS have been detected under various non-physiological conditions. They have been observed for the natural protein α-lactalbumin below pH 4.5 in the presence of a denaturant (Semisotnov, 1991) and for cytochrome *c* below pH 3 at chloride concentrations greater than 0.1M (Stellwagen, 1975). As well, many *de novo* designed proteins that have been characterized are believed

to be in the molten globular state: α_4, and its derivatives, α_2B, α_2C, and α_2D (Betz and Bryson, 1995), SGP (Lee, 1997), coil-LL (Betz and DeGrado, 1995), octarellin III (Houbrecht *et al.*, 1995).

The current challenge in *de novo* design is to achieve nativeness. With evidence of a hydrophobic core (see chapter 3) we know that MB-1 has achieved a folded state. The next step is to check the compactness of the fold. We can detect a loose fold by using a dye that will bind to hydrophobic regions on the structure, or exposed regions in the core.

1-Anilino-8-napthalene-sulfonic acid (ANSA) and similar derivatives are frequently used as non-covalent labels for proteins and membranes. These probes are essentially non-fluorescent in water, but are highly fluorescent when dissolved in non-polar solvents or when bound to macromolecules (Stryer, 1989), as shown in Figure 17.

ANSA has been used extensively as an extrinsic fluorescent probe to monitor conformational changes in biological macromolecules (Chang *et al.*, 1994), and to detect loosely folded, highly fluid structures (Itzhaki *et al.*, 1994). ANSA binding is highly effective for identifying proteins in a partly folded state, such as molten globules (Englehard *et al.*, 1996). The interest in binding information as well as the degree of enhancement in the fluorescence signal is due to the important role the packing of the hydrophobic core plays in the stabilization of the structure of the protein. ANSA will bind to any accesible hydrophobic clusters. The presence of exposed hydrophobic regions is known to decrease proteolytic stability. Thus in order to understand and modify MB-1's marginal resistance to proteases, we have to address the question, of whether there are exposed hydrophobic clusters in MB-1.

4.1.1 Objectives

The objectives of this chapter are:

- To determine if MB-1 molecule binds ANSA at different extents of folding taking into account: the enhancement factor, the number of ANSA binding sites available per molecule, and the dissociation constant (K_D).
- To assess the nativeness of the fold, ANSA binding to MB-1 will be compared to that of other proteins.

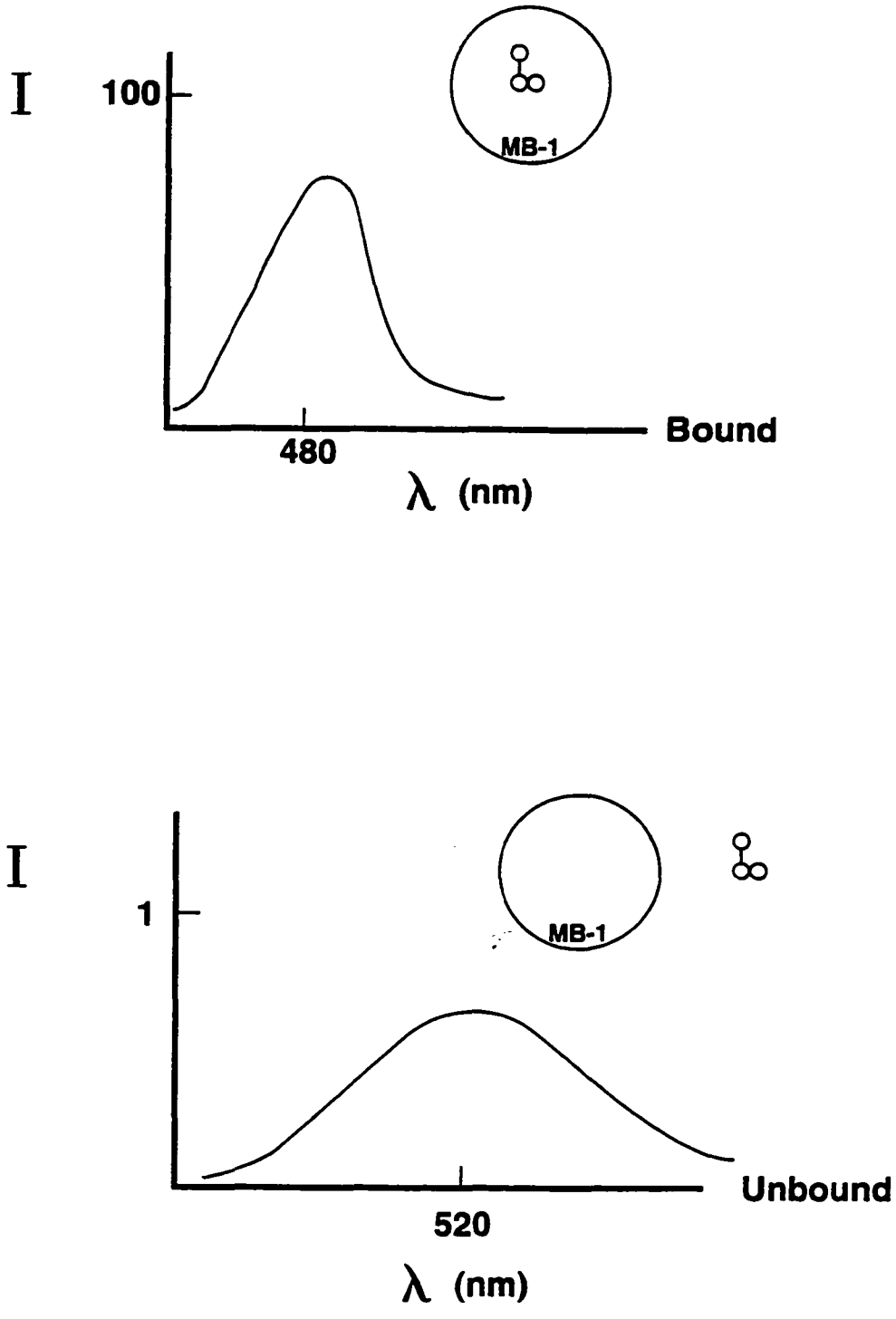


Figure 17: Spectra showing the emission of ANSA in the bound (A) and unbound (B) state. Maximal emmision in the bound state is around 480nm, and 520nm in the unbound state. Binding to a loosely folded protein leads to a large increase in ANSA fluorescence (top panel)

4.2 Materials and Methods

4.2.1 ANSA Binding to MB-1

MB-1 was denatured with urea at concentrations of 0-6M. The urea treated samples were left on the bench at room temperature (18-22 °C) overnight, to unfold the protein (a time study was performed to determine the time required for complete unfolding). ANSA was added to each of the samples at a concentration of 10 μM, mixed by pipette action and placed in the sample chamber. Samples were left to equilibrate for five minutes prior to recording emission spectra (Beauregard, 1996). All measurements were performed at 18 °C. ANSA was excited at 375nm and fluorescence was recorded from 475 to 530nm.

4.2.2 Determination of the Number of ANSA binding sites (n)

Two sets of ANSA studies were performed to obtain n, the number of binding sites on MB-1. (1) ANSA binding measurements were performed with constant protein concentration and varying ANSA concentration and, (2) with varying protein concentration at constant ANSA concentration. The first study was performed with a constant protein concentration of 0.2mg/mL and ANSA concentrations ranging from 0-400μM. Maximum ANSA concentration was determined by reaching saturation. ANSA was added from stock solutions prepared in 10mM acetate, pH 5.0. In the second study MB-1 concentration ranged from 0-1 mg/mL (also determined by reaching saturation) and ANSA concentration remained constant at 2μM. After addition of ANSA, the samples were equilibrated 5 min before measurements were made. These data were analysed using Scatchard plots (Scatchard, 1949).

4.2.3 Scatchard Plots

The change in ANSA fluorescence is used to evaluate the dissociation constant, K_D, for the MB-1- ANSA interaction. From study 1 described in the previous section, a plot of $1/\Delta F$ versus 1/[MB-1]μM was extrapolated to infinite protein concentration at 1/[MB-1]=0 and $1/\Delta F = 1/\Delta F_{max}$. The ordinate intercept of this plot gives an arbitrary unit, which when divided by the number of moles of ANSA reflect the increase in fluorescence in arbitrary units per μM ANSA bound to MB-1. From study 2, the data was plotted according to Scatchard's equation:

$$b/f = K_D n - K_D b$$

where b is equal to bound ANSA calculated using ΔF from study 2 divided by the arbitrary unit obtained in study 1; F is the amount of ANSA free in solution calculated by using total ANSA in solution t - b. When b/f is equal to zero, then $K_D n = K_D b$. K_D is related to the slope, where the slope is equal to $1/K_D$. The number of binding sites, n is obtained from the x-intercept of the plot (Scatchard, 1949)

4.3 Results

Figure 18 shows that the binding of ANSA decreases as the protein is unfolded with urea, which is to be expected, because when the protein is completely unfolded, it will no longer bind ANSA (Semisotnov, 1990). Maximal binding occurs at 0M urea, indicating that MB-1 binds ANSA in its "native" state. Fluorescence enhancement factor describes the increase in ANSA fluorescence upon binding to a hydrophobic region. The fluorescence enhancement factor upon binding to MB-1 was 2.5, a low value when compared to what is expected of *de novo* or MGS proteins. Fluorescence goes down as

the protein unfolds, indicating the absence of folding intermediates. If folding intermediates were present, there would be an increase in intensity of ANSA fluoresence. This would result in a peak at low concentrations of urea.

The Scatchard plot study, (Figures 19 a,b), show that MB-1 has an average increase of fluorescence of 5.6 arbitrary units per μM ANSA bound (Figure 19a). Using this arbitrary value, the number of ANSA binding sites (n) and the affinity with which it is bound (K_D) was obtained from the second plot (Figure 19b). We found that on average, MB-1 has 1.8 binding sites per molecule at 18 °C. The average K_D for ANSA bound to MB-1 was 11.7 μM.

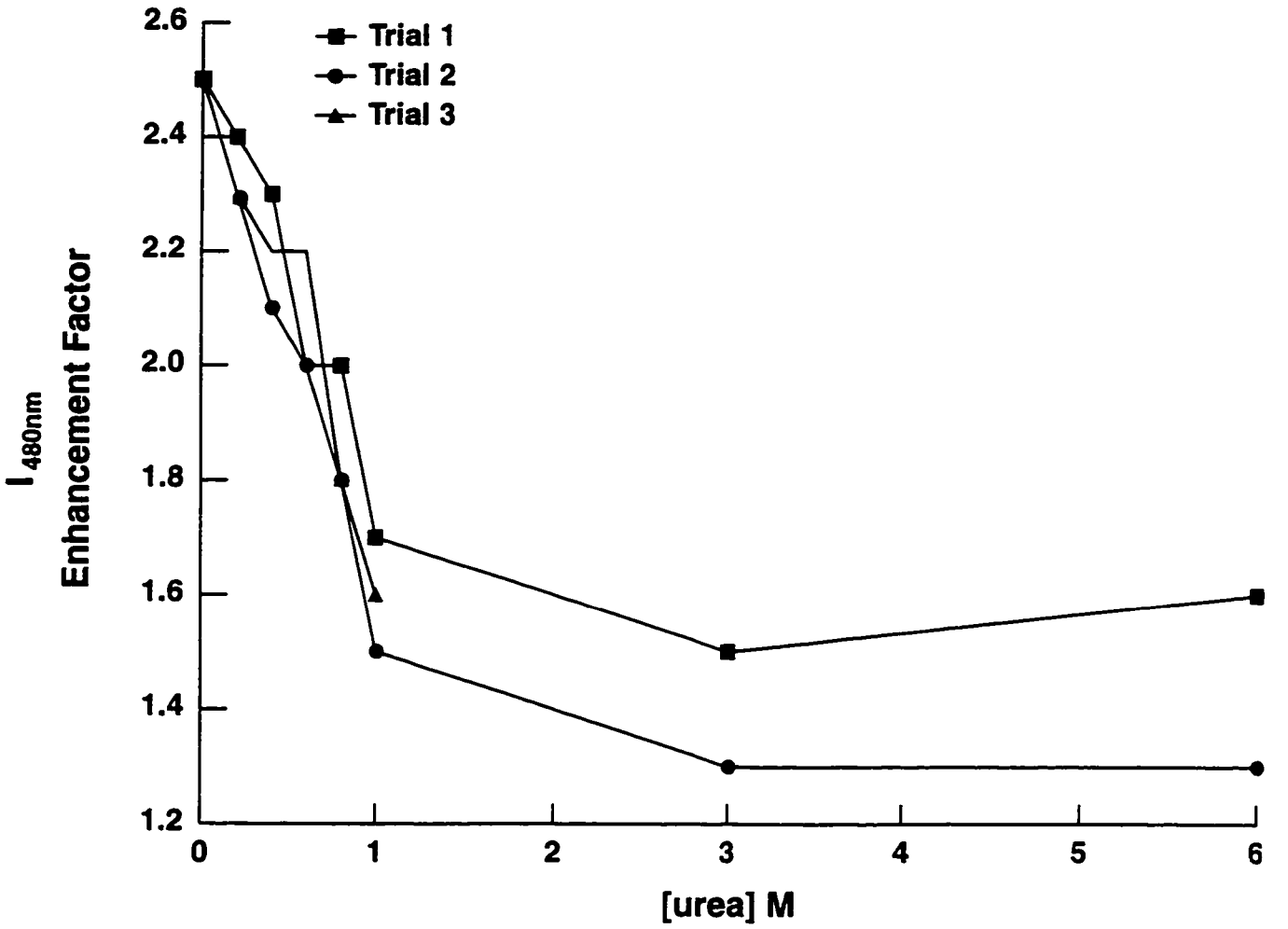


Figure 18: ANSA binding to MB-1. ANSA was used at a concentration of 10 μM, MB-1 was 0.2 mg/mL. MB-1 was unfolded using 0-6M urea. ANSA fluorescence intensity at 480nm was recorded and plotted versus [urea].

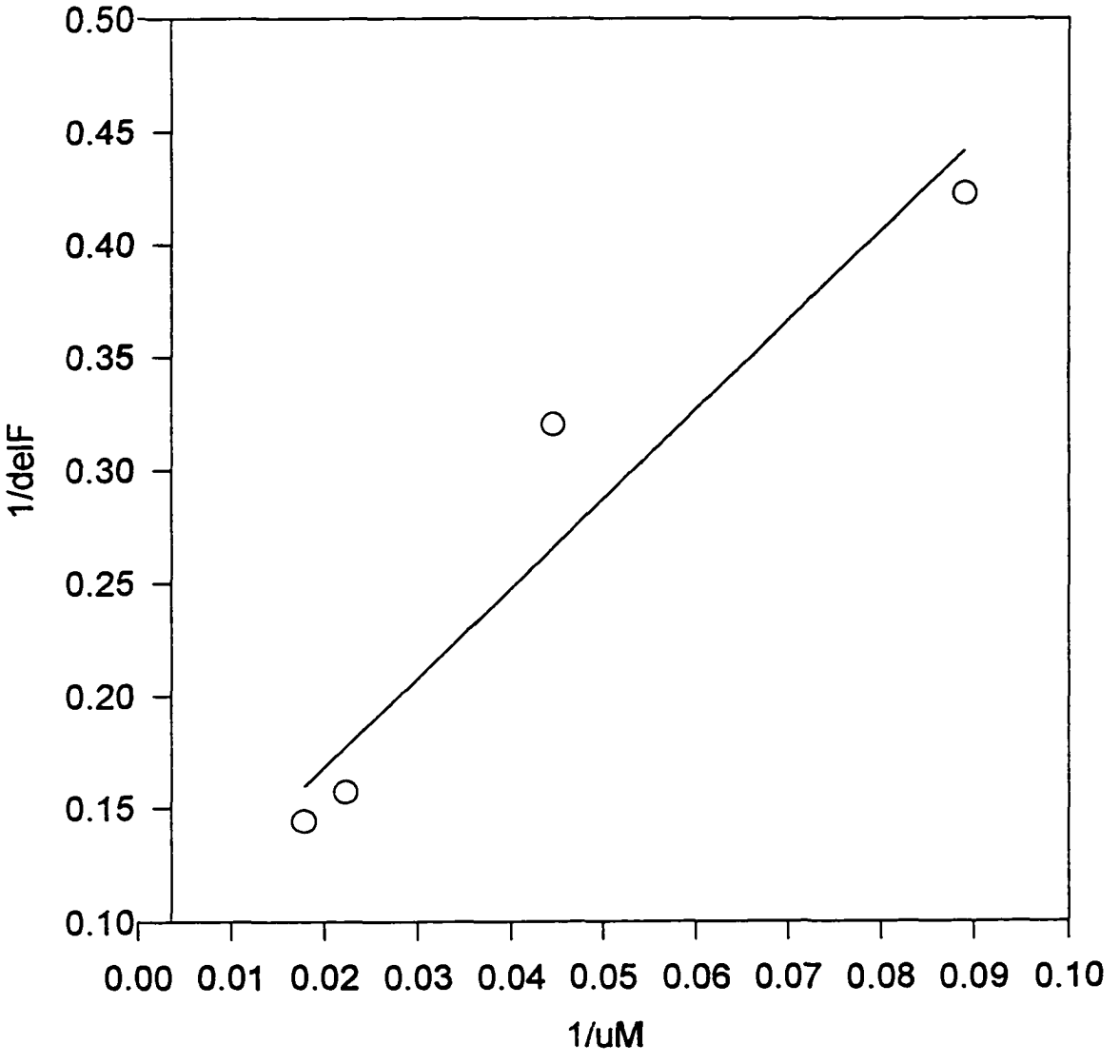


Figure 19a: Estimation of ANSA bound to MB-1. A double reciprocal plot of 1/fluorescence intensity versus 1/protein concentration was used to determine the maximum increase in fluorescence per μM ANSA bound. The ANSA concentration was 2μM and the protein concentration varied between 0-0.8mg/mL. Extrapolation to infinite MB-1 concentration indicates an ANSA fluorescence of 11.23 arbitrary units for binding with 2μM ANSA; i.e. an increase of fluoresence of 5.62 arbitrary units/μM ANSA bound.

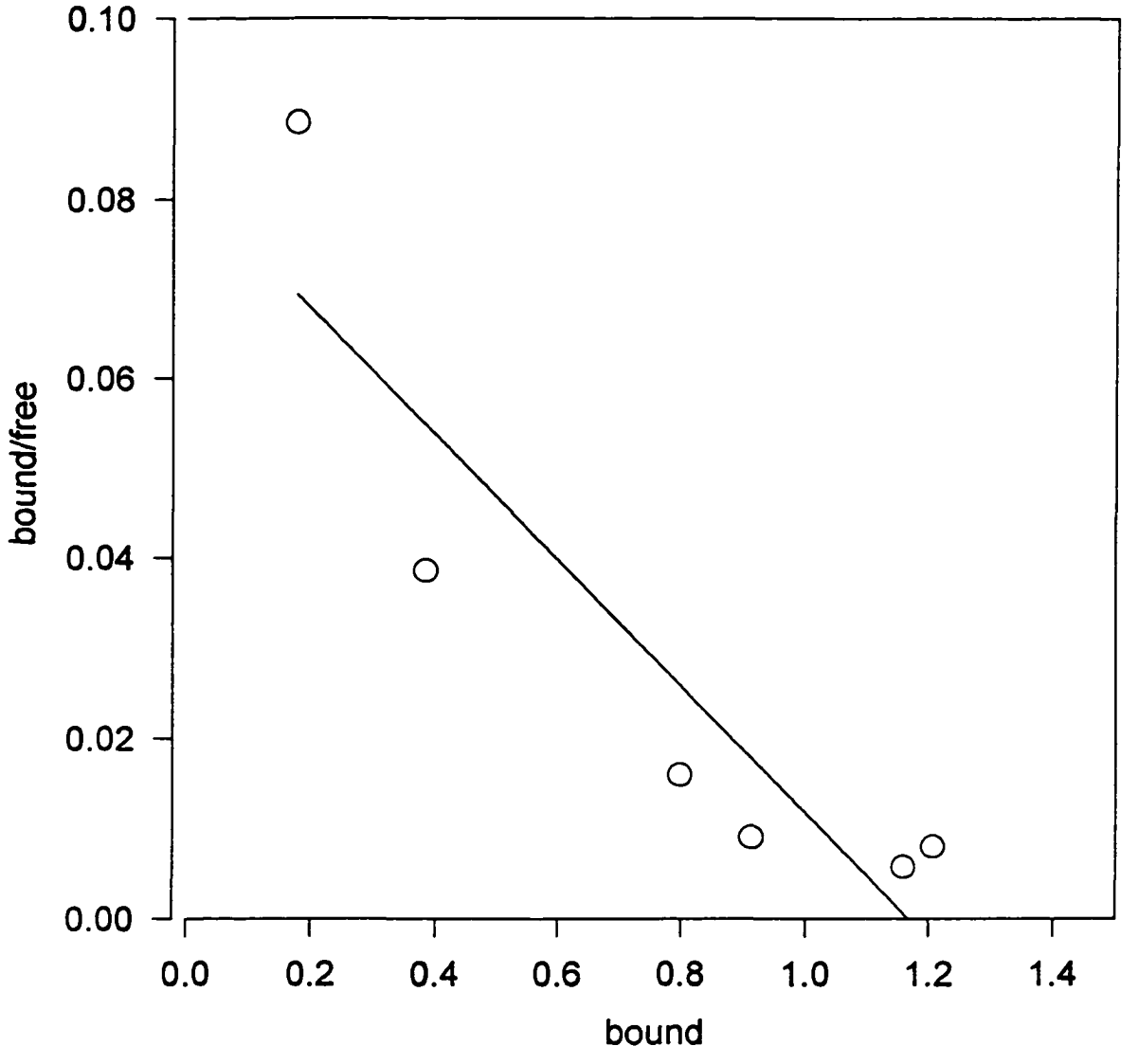


Figure 19b: Scatchard plot for the binding of ANSA to MB-1. The data of titration with ANSA are plotted according to Scatchard equation $b/f = K_D n - K_D b$, where b is calculated from ΔF assuming the value of ΔF for bound ANSA to be 5.62 arbitrary units per μM. We calculate f from f = t-b, where b is the fraction of bound ligand and t and f are the total and free concentration, respectively.

4.4 Discussion

The availability or exposure of hydrophobic clusters as found in proteins with fluid tertiary structure was probed using ANSA. To better illustrate how MB-1 compares with other proteins, ANSA binding parameters have been listed in Table I. In the first part of this table, inspection of the parameters for native proteins reveals that binding has very little impact on ANSA fluorescence (small enhancement factor). This also applies to ANSA λ_{max} which is typical for a polar environment (510nm). Thus, in native proteins, ANSA would not penetrate the core, as shown by its spectroscopic features. The second part of Table I lists binding properties of proteins submitted to destabilizing conditions. Such proteins have extensive secondary structure, but a highly fluid core that favors proteolytic degradation (called molten globule states or compact folding intermediate, see section 4.1). In this case the enhancement factors are very high, and the λ_{max} suggests that ANSA gets buried in a non-polar environment (i.e. 470-480 nm). Values of K_D and n are not very informative, as shown by a comparison between native and molten globule state proteins. The ability of some proteins to bind ANSA under non-destabilizing conditions may explain this.

Investigations done on *de novo* designed proteins are quoted in the bottom part of Table I. Looking at the enhancement factor of MB-1, it seems that this protein behaves more like a tightly folded, native protein, than an MGS or a loosely folded protein. Clearly, ANSA is excluded from its hydrophobic core, at variance with all the other synthetic proteins reported so far. The spectra measured with ANSA and MB-1 featured two peaks. The most important one (under conditions used for the unfolding study) was

at 504 nm, i.e. close to the value for unbound ANSA. Another peak was found at 474 nm, indicating some binding (spectra shown in Appendix F). Calculation of K_D for this binding revealed a rather high affinity. The evidence of ANSA binding (second peak, K_D, n) appeared difficult to recouncile with the obvious inability of MB-1 to enhance ANSA fluorescence emission like MGS do. Two hypotheses were considered in an attempt to understand this phenomenon.

As shown in figure 5b , one end of MB-1 exposes four hydrophobic side chain (M,A at the N-terminus, V,A at the C-terminus). Such a site would offer some affinity to ANSA, but would not allow for burial of its aromatic rings that would affect its fluorescence intensity. The terminii are in fact quite flexible.

Potentially, the presence of a small percentage of proteolytic degradation product in our MB-1 preparations may account for our results. This product is predicted to have three helices, having lost one. Such protein would not have an ideal core due to the truncation, and would accept ANSA in its fluid core. This scenario would explain the second peak of fluorescence: there would be two populations, made of 90% well folded MB-1, and 10% of destabilised, ANSA-binding, proteolytic product (percentages estimated on the basis of electrophoresis analysis, see Appendix E). This theory is also supported by spectral features of ANSA when MB-1 is present: two peaks were routinely found, one being at 504 nm.

Our results support the existence of a tightly folded core in MB-1 (at least for most of the protein population). Increased susceptibility to proteases in the rumen due to exposure of core residues or clusters can be excluded. While most ANSA appears to be

excluded from MB-1, there is a small population of a protein that binds to it. Further experiments are required to fully understand the contribution of degradation products to this phenomenon. Folding intermediates were not detected upon denaturation; this suggests that any oligomerization of MB-1 does not involve hydrophobic interactions. Early disruption of such interactions would have resulted in enhancement of ANSA fluorescence. Such phenomenon has not been detected here. Thus, when MB-1 associates at high concentration, it is probably not due to hydrophobic interactions.

Table I: ANSA Binding Parameters for MB-1 and Other Proteins

Protein (state/condition)	Enhancement Factor	Binding Sites	K_D (μM)	λ_{max} (nm)
NATIVE PROTEINS				
α-lactalbumin[1]	2.0	1	85-900	510
β-lactamase	9.0	2	40	480
carbonic anhydrase[1] (pH 8.0)	1.8	n/a	n/a	510
MGS Compact Intermediates				
α-lactalbumin (pH 2.0)[1]	70	n/a	n/a	479
carbonic anhydrase (pH 3.6)[1]	120	≥2	23	478
β-lactamase[1] (0.8GuHCl)	15	3	77	480
LDH A state[3]	7	n/a	n/a	482
Acetyl cholinesterase[4]	8	n/a	n/a	n/a
***DE NOVO* DESIGNED PROTEINS**				
MB-1	2.5	1.85	11.76	474, 504
α_2B[5]	100	n/a	50	n/a
α_2D[5]	10	n/a	n/a	n/a
SGP[6]	6	1.17	1.8	~475
Octarellin III[7]	100	n/a	n/a	n/a
Coil-VL[8]	1000	n/a	220	475
Coil-LL[8]	300	n/a	4	464

[1], Semisotnov, 1991; [2], Shi *et al.*, 1994; [3], Anderson and Weber, 1966; [4], Kreimer, 1994; [5], Raleigh *et al.*, 1995; [6], Lee *et al.*, 1997; [7], Houbrecht *et al.*, 1995; [8], Betz *et al.*, 1995
n/a: not available

5. Denaturation Studies: Structural Stability of the Tertiary Structure

5.1 Introduction

As discussed in section 1.4.2, MB-1 is not stable at rumen temperature having a Tm (melting temperature) of 39 °C (MacCallum *et al.*, 1997). The low Tm leads to increased proteolysis since when the protein is unfolded, or partially unfolded, it is more susceptible to attack by proteolytic enzymes (Goldberg, 1986). So it is important to evaluate folding thermodynamics of MB-1 in order to reassess our design and reveal strategies for improvements in stability. From the same earlier study, it was also discovered that the thermal unfolding of MB-1 was not reversible. Therefore, it was not useful to determine the change in free energy for folding-unfolding transitions (ΔG), or the change in heat capacity (ΔCp), two criteria for nativeness. The free energy for unfolding-folding transitions would predict the probability of MB-1 being folded as per design based on energetically stable conformations. The change in heat capacity can reveal information on the core of MB-1, allowing us to predict if it is sufficiently hydrophobic. These parameters would allow us to compare MB-1 to other native as well as other *de novo* proteins, and understand the behavior of MB-1 in solution. Unfolding by urea or guanidinium HCl in general is more likely to be completely reversible than thermal unfolding (Creighton, 1990) and therefore useful for our purposes.

Fluorescence is a powerful technique to detect the unfolding of proteins and to calculate its thermodynamic parameters (Jaenicke, 1996; Kwon, 1996; Stellwagen, 1978;

Pfeil and Privalov, 1976). In MB-1, fluorescence is emitted by the lone tyrosine which is known to be in the hydrophobic core of the protein as shown in chapter 3. When MB-1 is unfolded using a denaturant, its tertiary structure is disrupted, changing the environment of tyrosine from non-polar (core) to polar (buffer). This was shown in chapter 3 using fluorescence quenching. As the environment changes, the emission of tyrosine fluorescence should also change. By monitoring tyrosine emission, it is hoped that the transition from folded to unfolded state can be detected. This would allow us to obtain thermodynamic parameters. The ideal unfolding curve should have three parts: 1) the pre-transition region, which shows how the protein fluorescence is dependant upon addition of the denaturant in the native state, 2) the transition region, which shows how the fluorescence varies as unfolding occurs, and 3) the post-transition region, which shows how the fluorescence for the unfolded protein varies with the denaturant. All of these parts are necessary for thermodynamic analysis of protein unfolding curves (Pace *et al.*, 1988).

5.1.1 Objectives

For this chapter, the objectives are:

- To find an experimental parameter that will allow monitoring of unfolding.
- To determine experimental conditions that will allow construction of a reversible denaturation curve, containing all three parts necessary for analysis.
- If a proper curve is obtained, to extract ΔG and ΔCp. These two values will reveal information on folding stability and nativeness.

5.2 Materials and Methods

MB-1 was used at a concentration of 0.2mg/mL. Urea was added at a concentration range of 0-4M. After urea was added, the samples were left overnight on the bench (18-20 °C) to allow the protein to unfold. Samples were allowed to thermally equilibrate (to 18 °C) for 5 minutes in the fluorometer sample chamber before a measurement was recorded. Buffer blanks were also measured for each concentration of urea and measurements were appropriately corrected using these values. Excitation and emission spectra were recorded as described in section 3.1.5.

Quenching studies were performed as per the protocol listed in section 3.1.5.

MB-1 digestion experiments were performed with the enzyme chymotrypsin. Chymotrypsin was chosen since it is known that it will cleave MB-1. Two MB-1 samples at 0.2mg/mL were used, both thermally equilibrated overnight, one at 4 °C and the other at 18 °C. Chymotrypsin was used at 2.5 units per 200μL sample. An aliquot was removed before adding the enzyme and was labelled "time zero". Subsequent aliquots were taken every 5 min over a 60 minute time period. Experiments were carried out at room temperature Analysis of data was performed by estimating rates of degradation by SDS-PAGE (staining was performed as per section 2.1).

5.3 Results

Urea unfolding experiments have been shown to be more reversible because they are done at a fixed temperature, selected in a way to promote the native conformation and to avoid thermally- induced aggregation (Creighton, 1991). Before the actual urea unfolding experiments were begun, it was important to find a suitable temperature range

over which MB-1 could be considered folded.

From chapter 3 results suggest that MB-1 is folded at 18 °C. If a lower temperature where MB-1 was folded could be detected, working at a lower temperature would minimize protein degradation during the experiments. We decided to repeat experiments done in chapter 3, i.e. fluorescence quenching with citrate. Measurements were made on the protein at 4 and 18 °C. By doing this, we could see if the protein is more or less folded at those temperatures based on the ability of the quencher to access the core. If the protein unfolds, then we should see more quenching of MB-1.

Results show that MB-1 fluorescence is more quenched at 4 °C than at 18 °C, indicating that the tyrosine is more exposed at 4 °C (Table 2, appendix C and D). MB-1 appears to unfold at 4 °C.

Cold denaturation of proteins above 0 °C is an unexpected phenomenon. This was further examined by performing digestion experiments at both temperatures. As previously mentioned in the introduction, proteins that are partially or completely unfolded are more susceptible to proteolytic degradation. To confirm MB-1 unfolding at 4 °C, digestion studies were performed on MB-1 with chymotrypsin, which will cleave MB-1 at its tyrosine. Digestion studies with chymotrypsin show that MB-1 is more stable at 18 °C than at 4 °C where it is more rapidly degraded (30-35 min and 20 minutes lifetimes respectively), confirming that it unfolds at this temperature (Appendix A and B).

Table II: Effect of Temperature on MB-1 Fold

Sample/Treatment	Quenching	Lifetime in presence of protease
MB-1 at 4 °C	14%	20 minutes
MB-1 at 18 °C	0.5%	30-35 minutes

Both of these experiments suggest cold denaturation of MB-1 occurs at 4 °C, in accordance with preliminary circular dichroiism measurements (Morrison and Szabo, unpublished). Thus it appears that the experiments have to be done at 18 °C, where the protein seems to more folded.

Once the optimal temperature range was established an attempt was made to find a suitable parameter to monitor unfolding. A way to perturb tyrosine when the protein was unfolded was needed. Results shown here and in chapters 3 and 4 show that the environment of tyrosine was sufficiently disturbed in order to observe a change in I_{302nm} when MB-1 unfolds. However, the use of high concentrations of citrate (the quencher) results in a high ionic strength in the protein environment. Thus, thermodynamic analysis of data obtained in the presence of high citrate concentrations may not reflect thermodynamics of MB-1 unfolding *in vivo*. For those reasons, the experiment was performed without quencher, and emission at I_{302nm} was recorded. It was hoped that tyrosine emissions would change sufficiently to reveal a change upon unfolding.

MB-1 was unfolded using increasing concentrations of urea while tyrosine emission (I_{302nm}) was monitored. Figure 20 shows that as the concentration of urea increases and the protein is unfolding, the change in fluorescence is minor and, does not show any of the three expected regions (pre-transition, transition, and post-transition). It seems that the environment of tyrosine is not sufficiently disrupted to reveal a difference in its fluorescence yield. This study revealed that I_{302nm} was not a suitable parameter with which to monitor unfolding of MB-1.

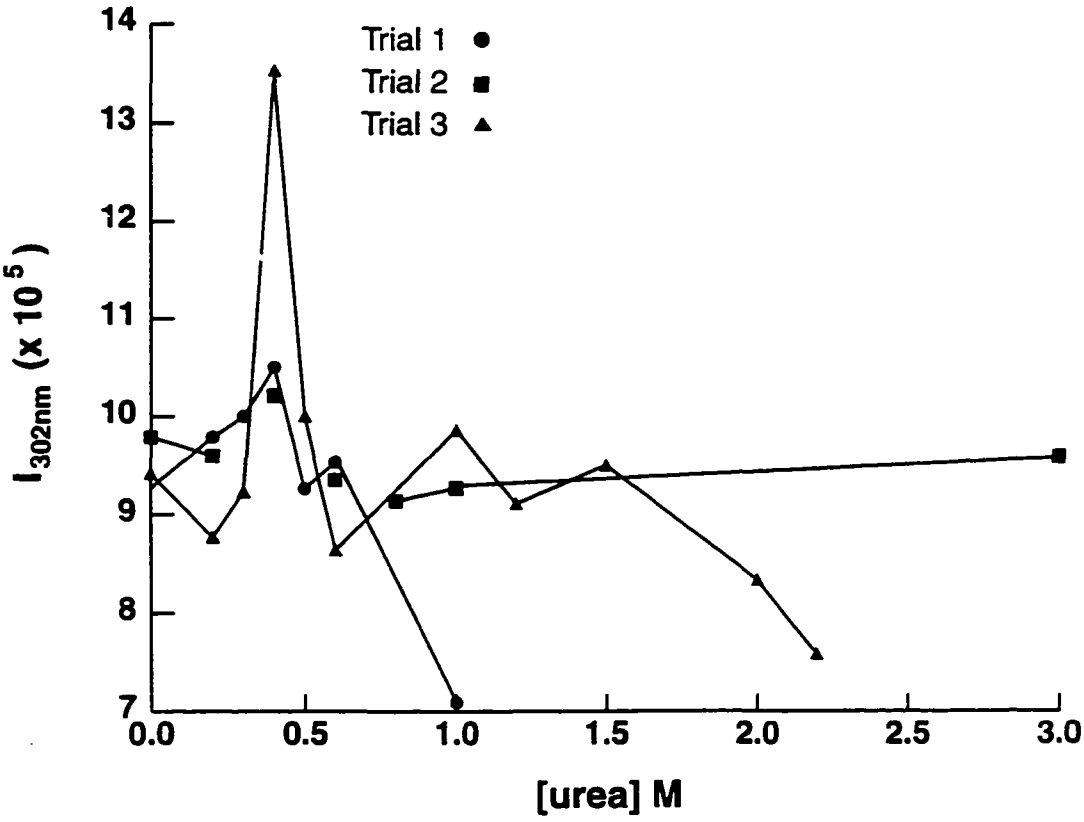


Figure 20: Unfolding of MB-1 using urea at different concentration. Fluorescence intensity at 302nm was found to be mostly unaffected by unfolding.

5.4 Discussion

Tests on MB-1 reveal that it is indeed folded at 18 °C, and reversible unfolding can be done at this temperature. Attempts at using tyrosine emission did not yield a curve containing the three important responses; pre-transition, transition, and post-transition. One explanation for this is that tyrosine may be hydrogen bonded to some other protein residues when the protein is folded, and to water when the protein is unfolded. This means that in future attempts, citrate quenching will have to be used (with the associated problems, i.e. high ionic strength), or a new technique must be found. Preliminary studies by Jennifer Morrison and Dr. Art Szabo using circular dichroism measurements are promising. These measurements can be done under low ionic strength, and are sensitive to unfolding. These experiments monitor changes to structure as conditions of the protein's environment are altered.

We were able to observe that MB-1 unfolds at 4 °C, aprocess termed cold denaturation. This phenomenon has been predicted to occur below 0°C for natural proteins (Privalov, 1990). MB-1 is one of the rare proteins that undergoes cold denaturation above 0 °C. This might be a feature of *de novo* designed proteins. It was predicted that proteins with a low Tm (like MB-1) would have a high cold denaturation temperature (Privalov, 1990). We provide here (one of the few reported) evidence that corroborates Privalov's predictions.

6. General Discussion

MB-1 was designed for the purpose of enhancing the essential amino acid pool available for dairy cattle. However problems from the first round of design must be addressed before the use of MB-1 can proceed. MB-1 has been shown to have low thermal stability and is predicted to be highly susceptible to rumen proteolytic degradation (MacCallum, 1997). Low stability and low resistance to proteases can be attributed to a loose or misfolded structure (Goldberg, 1986; Liao, 1993). MB-1's primary and secondary structure are known and appear compatible with the target structure for MB-1 (Beauregard *et al.*, 1995). Thus, MB-1 instability could arise from tertiary structure misfolding or a lack of compactness. The objective of this thesis was to characterize the tertiary structure of MB-1. Findings from these experiments, provide information to reassess and improve MB-1's design.

To do this, three main points were addressed: (1) the position of tyrosine was verified, (2) the fluidity of the core was measured, (3) and a study of the thermodynamic parameters was attempted. Each of these studies revealed information on the tertiary structure of MB-1.

6.1 Verification of the Position of Tyrosine 62 in MB-1 using Fluorescence Quenching

Citrate is a polar, charged quencher and is not expected to penetrate a hydrophobic core. Citrate quenching of L-tyrosine is both dynamic and static (Leavis and Lehrer, 1978). Dynamic quenching is a result of diffusion of the quencher to the

fluorophore during the lifetime of the excited state. Static quenching involves the formation of a non-fluorescent ground state complex made up of the quencher and the fluorophore (Lakowicz, 1983). These two components were not considered seperately, but rather as one. Both static and dynamic quenching were observed on unfolded MB-1.

By showing that the quenchers could not access tyrosine when MB-1 was in the folded state, we showed that tyrosine was not exposed to the outside aqueous environment; indicating that a core was formed. The lone tyrosine was indeed protected from polar quenchers by the core. This study revealed that the design approach for MB-1 was successful: a hydrophobic region had been formed, and Y62 is buried in the core as per design.

That fluorescence quenching studies can provide insight concerning the dynamic nature of protein molecules was first realized by Lakowicz and Weber (1973). They found that O_2 was able to quench fluorescence of buried tryptophan residues. This was presumably due to the fact that proteins are not rigid structures, but that they continually undergo random structural fluctuations that facilitate the inward diffusion of quencher. Subsequently, it was found that acrylamide was also able to quench the fluorescence of buried residues in proteins such as ribonuclease T1 and aldolase (Eftink and Ghiron, 1975, 1976, 1977). If the quencher is very small, one can imagine that it might be able to permeate the protein matrix by diffusing through packing void spaces with very little relaxation of the protein required. This theory holds that some quenchers actually penetrate into the matrix of the protein. An alternative view is that a buried residue periodically becomes exposed to the surface of the protein as a result of local unfolding of

the protein (Eftink and Ghiron, 1980). The flexibility of proteins is ultimately the result of the fact that the intramolecular non-bonded interactions (i.e. hydrogen bonds, van der Waals interactions) that serve to maintain the globular state of a protein are relatively weak interactions (Eftink and Ghiron, 1980).

The acrylamide study showed quenching of the tyrosine in both the folded and the unfolded protein. This is normal behaviour for acrylamide which is known to penetrate hydrophobic cores (Eftink and Ghiron, 1975, 1976, 1977). No major differences were detected between quenching with acrylamide in the folded or unfolded state. This means that tyrosine fluorescence is not dependant on folding (as confirmed in section 3.3). This also confirms that the quenching obtained with citrate in the unfolded state comes from citrate quenching and not from the impact of the conformational changes on tyrosine that occurs during unfolding.

6.2 Assessment of Core Fluidity with ANSA

Once it was established that a core was formed, compactness of the fold was next assessed. This was achieved using ANSA binding to probe for any exposed hydrophobic regions or pockets. Less than three binding sites were discovered for MB-1, presumably formed by the hydrophobic residues located at the N and C terminii of the protein. The K_D value showed that ANSA binds with a high affinity (i.e. specificity), and not in a non-specific association that would indicate loosely exposed hydrophobic regions and thus a loose fold. The low enhancement factor obtained upon binding was also characteristic of more "native" proteins than molten globule states, indicating that ANSA was not inserting into a fluid core, but probably binding to a hydrophobic cluster. This means the protein is

compact.

ANSA binding is a commonly used method of characterization for the compactness of a fold (Shi, 1990; Lee, 1997; Betz, 1995). The act of binding ANSA does not indicate a molten globular state, as many native proteins bind ANSA in their folded state (Shi, 1995). More accurate information on the fold of a protein can be revealed by the manner in which the protein binds ANSA using parameters such as enhancement factor, affinity (K_D), number of binding sites (n), and λ_{max}. MB-1 has been shown to bind ANSA in a manner closely related to that of natural, folded proteins. The enhancement factor is very small as compared to proteins known to be in the molten globular state, indicating that the core of MB-1 is impenetrable to ANSA.

When MB-1 is compared to other *de novo* proteins (Table I), it is far more compact than its peers, as indicated by the low enhancement factor. This was a design goal at the beginning of the MB-1 design project. The compactness and packing of the core has long been recognized as an important factor in resistance to proteolytic degradation. Based on results presented in this thesis, it can be concluded that MB-1 improvements should not focus on packing and compactness of the core, but rather on other aspects of MB-1.

Other information was obtained on MB-1 associations. If MB-1 forms oligomers, then they would not be bonded by hydrophobic forces, but rather through electrostatic interactions (Chapter 4). MB-1 binds ANSA maximally at 0M urea, and as it unfolds, no folding intermediates are detected, as would be the case if the protein were held together by hydrophobic forces.

6.3 Denaturation Studies: Structural Stability of the Tertiary Structure

In the third part of this thesis, we attempted to determine a procedure that would allow for complete thermodynamic analysis of MB-1 unfolding. We attempted to monitor unfolding in the presence of urea, in the absence of quenching agent, and this approach was unsuccessful. Analysis of unfolding thermodynamics was not possible due to the lack of response in tyrosine fluorescence to unfolding. However, this "negative" result, led us to further insight into the environment of tyrosine. Based on the tyrosine response to unfolding (no specific change in intensity at I_{302nm}), it can be inferred that the tyrosine is possibly hydrogen bonded to a neighboring threonine in the core, and when it becomes unfolded it is possibly hydrogen bonded to water or another amino acid.

The temperature dependance study that was performed revealed that MB-1 was more unfolded at 4 °C. This is an exceptional phenomenon, most likely a feature of *de novo* design. Cold denaturation is predicted for all proteins at temperatures below 0 °C. Our results verify a prediction made by Privalov, (1990), that states that proteins with a low Tm (denaturation temperature) will generally have a higher Tc (cold denaturation temperature). MB-1 has a low Tm at 39 °C, and in agreement with Privalov's theory, it also has a high Tc (cold denaturation temperature).

6.4 Comparison of MB-1 to Other *De Novo* Designed Proteins

Apart from MB-1, a number of proteins have been designed (for uses other than nutrition) by different groups. Several *de novo* design projects have achieved the desired folding of proteins but do not demonstrate all the properties of natural proteins (Handel *et al.*, 1993; Regan *et al.*, 1988; Hecht *et al.*, 1990; Raleigh *et al.*, 1992; Choma *et al.*, 1994;

Fezoui *et al.*, 1994).

Most *de novo* designed proteins characterized to date show a high degree of secondary structure and very little tertiary structure, which is characteristic of the 'molten globule state' (Betz *et al.*, 1993). Molten globule state proteins have a poorly defined, somewhat dynamic hydrophobic core, which would account for their divergence in structure from natural proteins. Results reported by Munson *et al.* (1996) suggest that the hydrophobic core is critical for the stability and structural properties of a protein. By repacking the core of the *de novo* designed protein *Rop*, Munson *et al* were able to produce stable and non-stable mutants based on amino acid choices for the hydrophobic positions.

De novo designed proteins exhibit some of the features of the molten globule, such as marginal stability, low cooperativity of unfolding, ANSA binding, and poor NMR resolution characteristics (Reagan and DeGrado, 1988; Semisotnov et al., 1990; Kametekar *et al.*, 1993; Quinn *et al.*, 1994; Raleigh *et al.*, 1995). Biophysical analysis of *de novo* designed helix-bundle proteins with four amphiphillic α-helices (like MB-1) have shown that they are like natural proteins but lack a well-ordered interior, making them more like molten globules (DeGrado *et al.*, 1988; Lutring and Chmielewski, 1994). This does not seem to be the case with MB-1. Results from thi study suggest that MB-1 has a well formed hydrophobic core, protecting tyrosine, as per design. There is also very specific ANSA binding to MB-1, presumably in the hydrophobic pocket created by the N and C terminal. There does not seem to be any penetration of ANSA in the core as shown by the small enhancement factor that was obtained.

All soluble globular proteins that have been characterized to date possess interiors rich in non-polar amino acids and surfaces rich in polar ones. This hydrophobic effect has a major effect on folding in *de novo* proteins. Clustering of non-polar amino acids is favoured thermodynamically and significantly stabilizes the fold (Kamtekar *et al.*, 1995). MB-1 compares well to other *de novo* projects having achieved a compact impenetrable hydrophobic core.

6.5 Conclusion

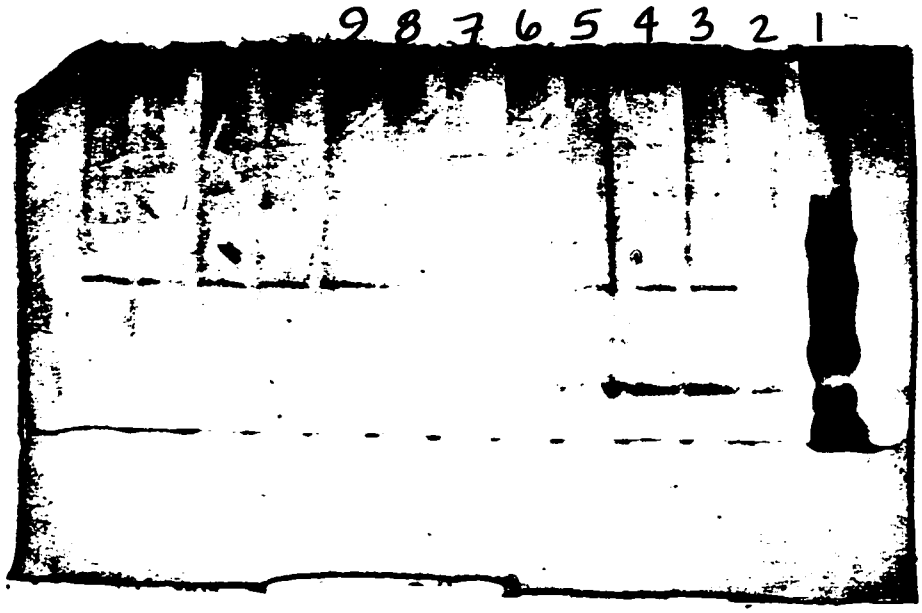
MB-1 was designed to adopt a specific fold, the α-helical bundle. Evidence presented in this thesis confirms that MB-1 is compact and folded as per design. From all the results presented here, the demonstrated low proteolytic resistance of MB-1 to Pronase E and Neutrase (MacCallum *et al.*, 1997) is not due to absence of a compact folded core. MB-1 has a compactly folded core, which exhibits behaviour characteristic of natural proteins. There was no indication of exposed regions within the core (there may however be one at the N and C terminal). Therefore the most likely cause of susceptibility to degradation is the low thermal stability of MB-1. As reported in section 1.5.2, the protein is 50% unfolded at the rumen temperature of 39 °C.

The next step in this project would be the completion of the thermodynamic study, in order to obtain the ΔCp value that would tell us if the protein is sufficiently hydrophobic. Mutations yielding more hydrophobicity would involve trytophan. Inserting a tryptophan into the protein instead of the tyrosine would be useful as (1) tryptophan is more hydrophobic than tyrosine, and (2) trytophan would allow for better methods of characterization, such as size exclusion chromatography, which could not be

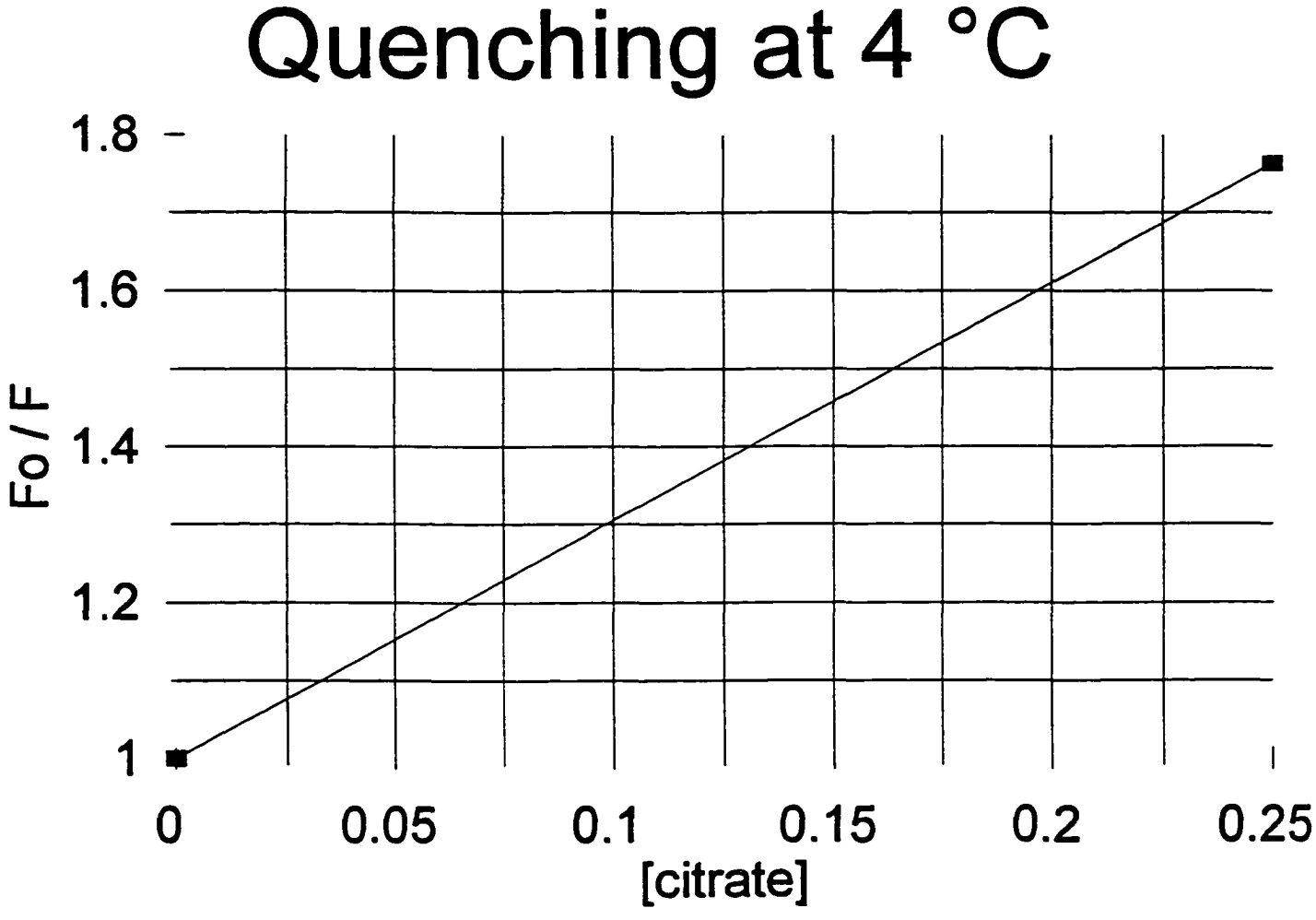
achieved due to weak tyrosine absorption coefficient. Another way of increasing stability would be to add fold specifiers, such as a disulphide bridge and β-branched amino acids in the core. A decrease in the hydrophobicity of the N and C terminal, could be done by changing the amino acids at the terminals. Another problem could be the presence of target sequences recognized by the proteases extracts used for the study (Pronase E and Neutrase). MB-1 could be tested with proteases extracted from the rumen to verify this hypothesis.

Appendix A: SDS-PAGE for the digestion studies with chymotrypsin at 4 °C. Lane 1 contains molecular weight markers (top down: 37.5, 29.2, 17.8, 9.0, 4.4 kDa), Lane 2 sample before experiment, Lane 3 time 0, Lane 4 time 2 min, Lane 5 time 5 min, Lane 6 time 10 min, Lane 7 time 15 min, Lane 8 time 20 min (note sample is now completely degraded).

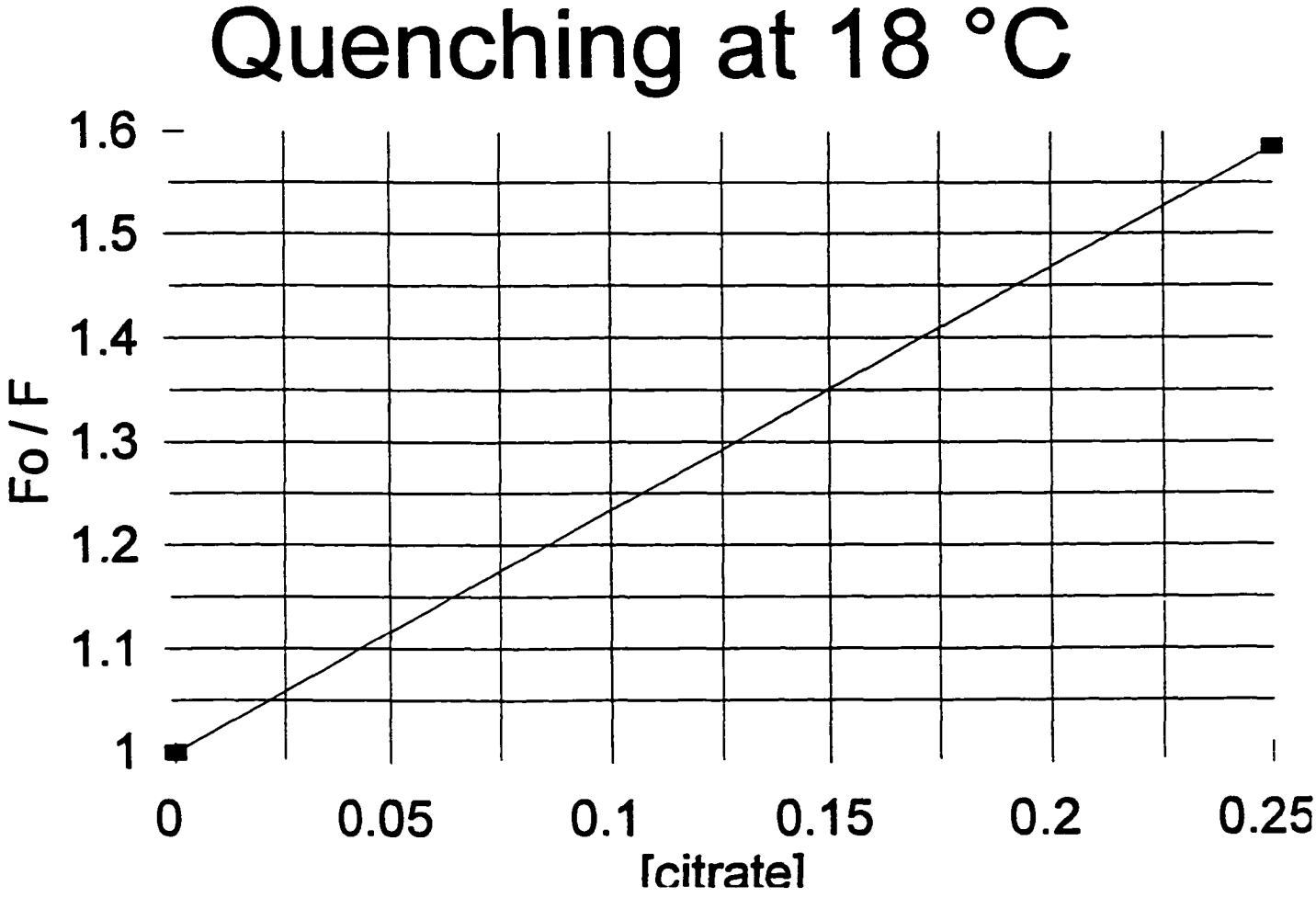
Appendix B: SDS-PAGE for the digestion study with chymotrypsin at 18 °C. Lane 1 contains molecular weight markers: (top-down: 37.5, 29.2, 17.8, 9.0, 4.4 kDa), Lane 2 sample before experiment, Lane 3 time o, Lane 4 time 2 min, Lane 5 5 min, Lane 6 time 10 min, Lane 7 time 15 min, Lane 8 time 20, Lane 9 time 25 (note that the gel is MB-1 is still visible but faded).



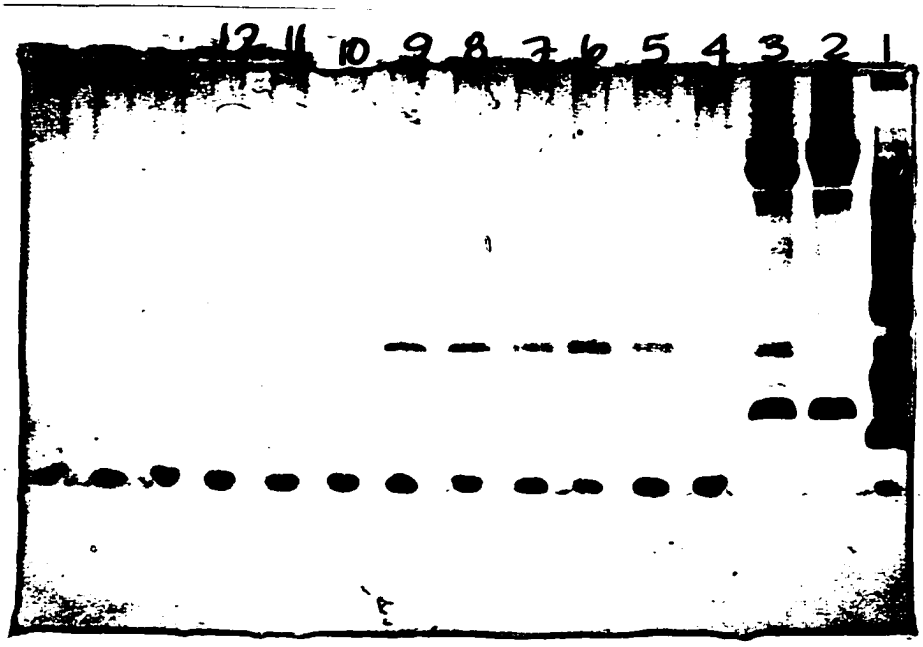
Appendix C: Stern-Volmer plot of results of quenching with citrate at 4 °C (plot shows the average of 5 trials).



Appendix D: Stern-Volmer plot of the citrate quenching data at 18 °C (plot shows the average of 5 trials).



Appendix E: SDS-PAGE showing the MB-1 band (11kDa) and the proteolytic degradation product (8kDa). Lane 1 contains molecular weight markers (top-down: 37.5, 29.2, 17.8, 9.0, 4.4 kDa), Lane 2 Sample before cleavage, Lane 3 Sample after cleavage, Lane 4 empty, Lanes 5-10 fractions from DEAE sepharose column. Note the presence of an 8kDa degradation band.



Appendix F: Spectrum of ANSA fluorescence in the presence of MB-1

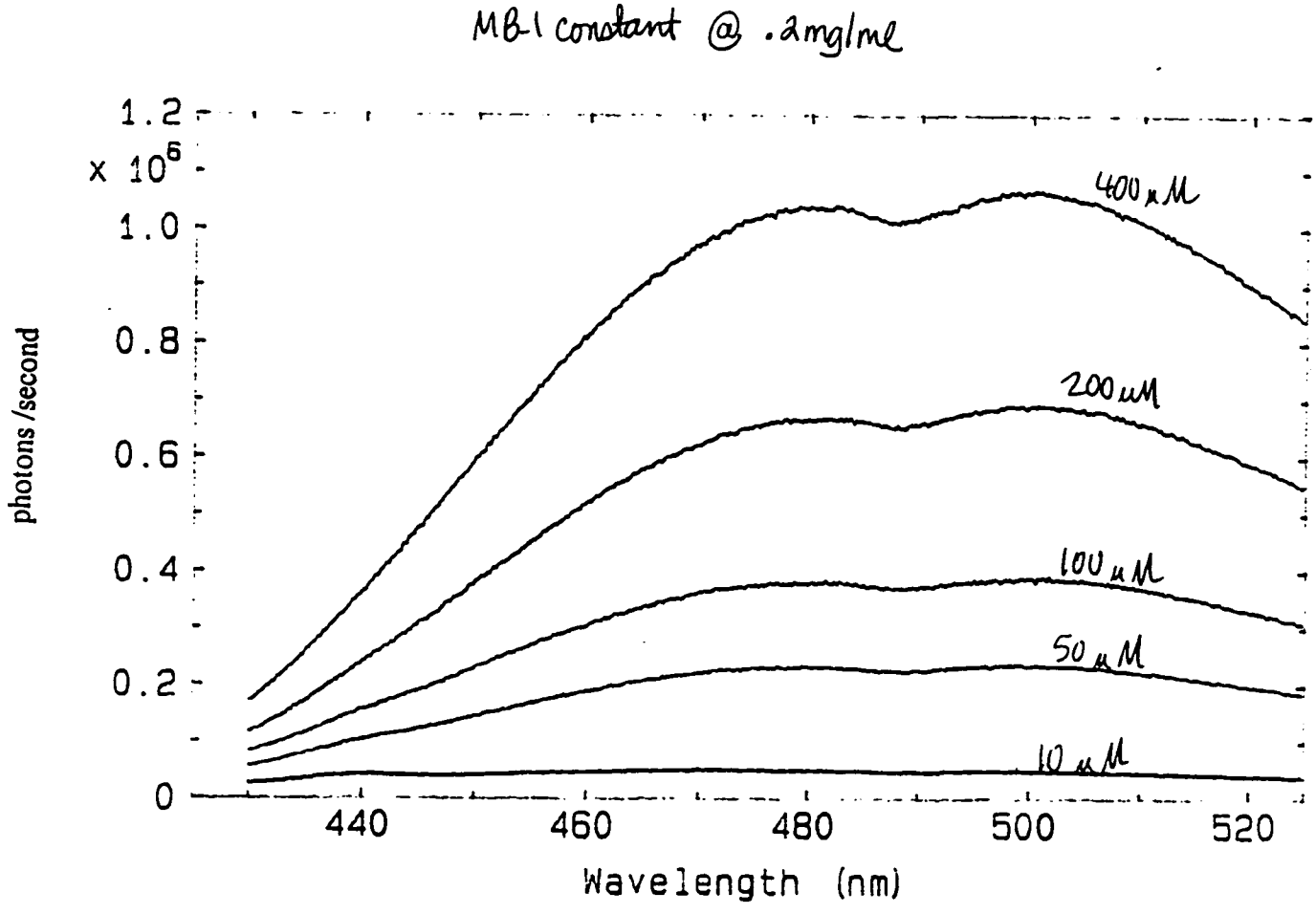


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IMAGE EVALUATION
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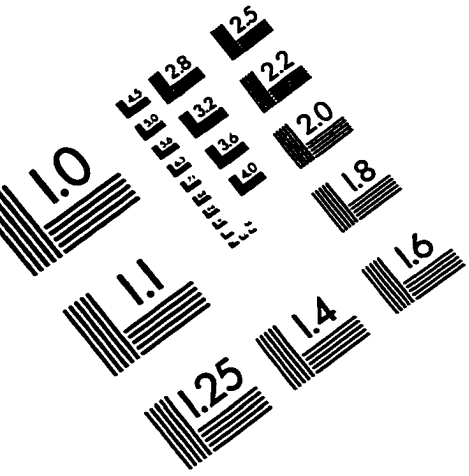

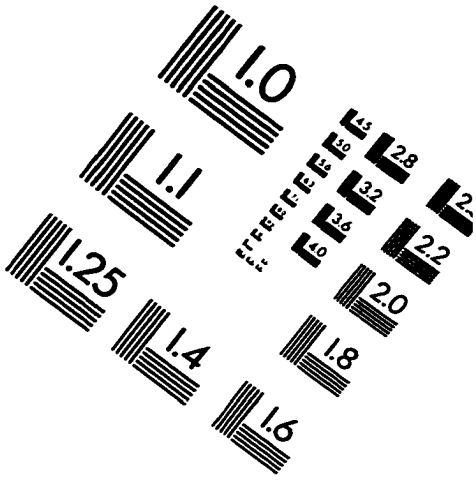

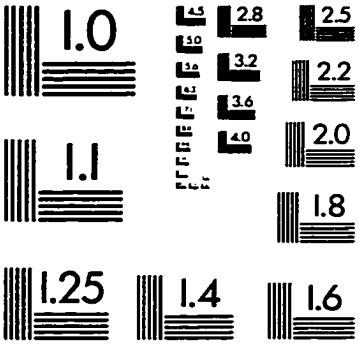

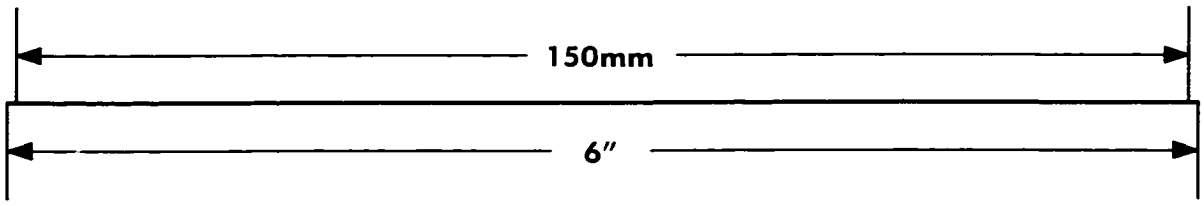

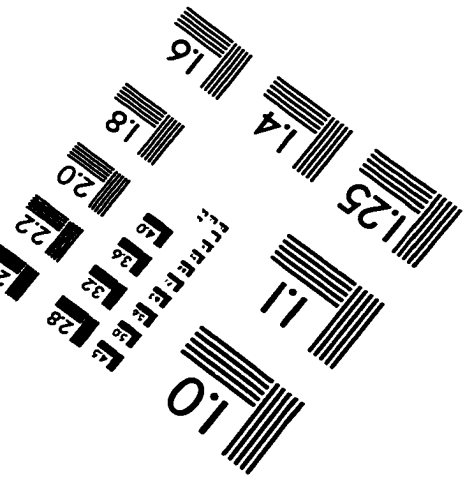

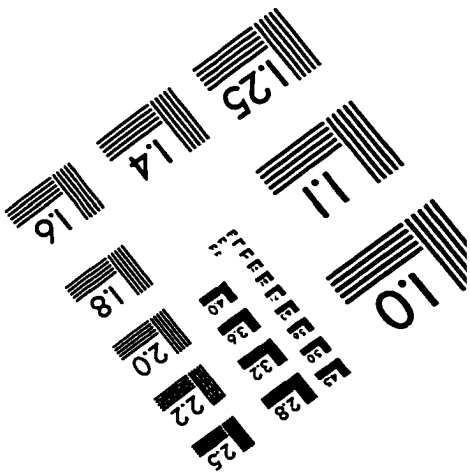

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