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Thermal denaturation of RNase A in aqueous-methanol solvent in the presence of urea

Pi-ju Chen
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**THERMAL DENATURATION OF RNASE A IN AQUEOUS-
METHANOL SOLVENT
IN THE PRESENCE OF UREA**

**A Thesis
Presented to
The Faculty of the Department of Chemistry
San Jose State University**

**In Partial Fulfillment
of the Requirements for the Degree
Master of Science**

**by
Pi-ju Chen
December, 1996**

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ABSTRACT

Thermal Denaturation of RNase A in Aqueous-Methanol Solvent in the Presence of Urea

by Pi-ju Chen

To characterize the partially folded intermediates along folding pathway, the thermal denaturation of RNase A in 50% (v/v) methanol solvent in the presence of urea at pH* 3.0, 0.1 M KCl was examined. Concentrations of urea were 0, 0.5, 1.0 and 1.5 M. The results show that the transition midpoint (T_m) decreases as the concentration of urea increases. T_m values obtained from fluorescence measurements are lower than those obtained from the corresponding circular dichroism measurements. The results indicate that secondary structure is more stable than tertiary structure in aqueous-methanol solvents. Further, urea destabilizes secondary structure to a greater degree than tertiary structure. This suggests that urea destabilizes the structure by competing for hydrogen bonds rather than through hydrophobic interactions. The loss of the tertiary and secondary structures are not coincident suggests the existence of a molten globule state.

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LIST OF ABBREVIATIONS

1. ϵ : Extinction Coefficient
2. $\Delta\epsilon$: Change in Extinction Coefficient
3. $G_{\text{denatured}}$: Gibbs Free Energy of the Unfolded State
4. G_{native} : Gibbs Free Energy of the Native State
5. ΔG_{unfold} , ΔG : Change in Gibbs Free Energy of Unfolding Process
6. ΔH : Change in Enthalpy
7. ANS: 1-anilinonaphthalene-8-sulfonate
8. b : Pathlength
9. BPTI: Bovine Pancreatic Trypsin Inhibitor
10. CD: Circular Dichroism
11. CSA: 10-camphorsulfonic Acid
12. DSC: Differential Scanning Calorimetry
13. F_u : Fraction of Unfolded Molecules
14. GdmCl: Guanidinium Chloride
15. I: Intermediate State
16. k : Boltzmann's Constant
17. K_{eq} : Equilibrium Constant
18. MeOH: Methanol

- 19. MW: Molecular Weight
- 20. N: Native State
- 21. NMR: Nuclear Magnetic Resonance
- 22. pH*: Apparent pH
- 23. pI: Isoelectric Point
- 24. R: Ideal Gas Constant
- 25. RNase A: Ribonuclease A
- 26. RNase T1: Ribonuclease T1
- 27. T: Absolute Temperature
- 28. Temp: Temperature
- 29. T_m: Transition Midpoint for Thermal Denaturation
- 30. U: Unfolded State
- 31. UV: Ultraviolet
- 32. v/v: Volume to Volume Ratio
- 33. w/v: Weight to Volume Ratio
- 34. y: Signal Amplitude
- 35. y_D: Signal Amplitude of the Denatured State Where y Is Measured
- 36. y_N: Signal Amplitude of the Native State Where y Is Measured

Chapter 1: INTRODUCTION

1.1 Protein Structure

Proteins are heteropolyamide polymers made up of twenty different α -amino acids. Different amino acids are distinguished from each other by their different side chains which attach to the fourth valency of the α -carbon. The 20 different side chains specified by genetic code contain a remarkable collection of diverse chemical groups. These form the vocabulary that allow proteins to exhibit such a great variety of structures and properties. The functional properties of proteins depend upon their three-dimensional structures. The three-dimensional structure arises because particular sequences of amino acids in polypeptide chains fold to generate specific three-dimensional structures from the linear chains. In other words, the sequence of amino acids defines its local structure which, upon further folding, defines higher order structures. Thus, the functions of proteins can be understood in terms of their structure, the three-dimensional relationship between the component atoms (Creighton, 1990).

The three-dimensional structures of protein molecules are organized in a “structural hierarchy.” The primary structure is the linear amino acid sequence, the order of amino acids along a polypeptide chain. The secondary structure is a

local arrangement of the polypeptide backbone which is mainly stabilized by hydrogen bonds between amide carbonyls and amide hydrogens. Common secondary structures include α -helix, β -sheet and turn. The tertiary structure refers to the packing of secondary structural elements relative to one another. This three-dimensional structure is essential to the biological activity of the protein. Some proteins are composed of two or more independent polypeptide chains known as subunits. Each polypeptide chain forms its own tertiary structure. The quaternary structure of proteins refers to the spatial arrangement of these subunits (Branden and Tooze, 1991).

1.2 Significance of Protein Folding

Polypeptide chains fold up into discrete, highly organized, and tightly packed three-dimensional structures. Both the patterns of the folded chains and the resultant overall shapes are diverse and complex. Proteins play an enormous variety of roles in biological systems. The enormous variety of amino acid sequences confers on proteins their powers of recognition and selectivity, catalysis, and specificity.

The fact that proteins with a given amino acid sequence always attain the same native structure and different sequences lead to different structures indicates that the amino acid sequence “codes” for native structure. However, how the

sequence determines how the polypeptide folds into its three-dimensional native structure is a major unsolved problem in biology and chemistry.

Knowledge of a protein's native structure is a prerequisite for the complete understanding of its function. Unfortunately, in spite of recent significant technological advances in X-ray crystallography and nuclear magnetic resonance (NMR), the experimental determination of native structure has been slow when compared with the rate of accumulation of amino acid sequence data. This makes the protein folding problem, the successful prediction of a protein's native structure from its amino acid sequence, central to rapid progress in protein engineering and design.

The ability to design *de novo* proteins requires an understanding of how sequences control folding. The problem of designing an amino acid sequence to adopt a desired three-dimensional structure has been addressed recently. The most successful *de novo* designs have been those of compact monomeric four-helix bundle proteins (Regan and DeGrado, 1988; DeGrado et al., 1989; Hecht et al., 1990). The proteins were shown to be highly helical and remarkably stable to denaturation by guanidinium chloride. Richardson and Richardson (1990) also have designed a four-helix cluster protein known as "felix." Remarkable advances like these and recent progress in genetic engineering may, one day, enable one to design a protein with a particular structure and function. An obvious advantage of

de novo designs for the study of protein structure is the possibility of examining the effect of structural features individually.

Knowledge of the protein folding code would also provide insight with regard to protein stability and thus allow for the engineering of proteins which are more stable than their naturally occurring counterparts. The increased stability of proteins would provide a longer shelf life and thus would be useful in pharmaceutical chemistry and food process industries.

1.3 Conformational States of Proteins

In protein folding or unfolding, different protein conformations differ only in the orientation about the ϕ and ψ angles (rotation angles) of the backbone and amino acid side chains. There are three conformational states of proteins which have been examined in detail: the native state, the unfolded state, and the compact intermediate state (Creighton, 1990).

1.3.1 The Native, Fully Folded State (N)

The native conformations of many proteins are known in great detail from the structures determined by X-ray crystallography and NMR studies. To be biologically active, every protein must maintain a unique three-dimensional structure. The most conspicuously similar aspects of native protein structures are the general conservation of the nonpolar character of the side chains that comprise

the folded interior, and the general prevalence of hydrophilic side chains at the surface (Perutz et al., 1965). Folded proteins demonstrate varying degree of flexibility. Flexibility is greatest at the protein surface, whereas it is least in the interior, even though side chain rotations occur. For example, the aromatic rings of most buried tyrosine and phenylalanine side chains are flipping by 180° on the millisecond time-scale (Wagner, 1983). In many cases, biological function requires flexibility of functional groups, chain segments, or domains (e.g. oxygen binding to myoglobin or hemoglobin).

The native structure is highly ordered and more stable and compact than the unfolded “random” polypeptide chain at ambient temperatures. Most of the interactions within the folded state are more favorable energetically than the corresponding interactions in the unfolded state under physiological conditions. These interactions, therefore, contribute to the net stability of the folded protein and make the fully folded state a unique conformation.

1.3.2 The Unfolded State (U)

The fully unfolded protein is a random coil in which the rotation angle about each bond angle along the backbone and side chains is independent of every other bond angle distant in the sequence, and is determined only by the local stereochemistry of the polypeptide backbone. Steric repulsions are significant between atoms close in the covalent structure and restrict the local flexibility.

Besides these constraints, the fully unfolded state has no definite structure and a very labile fluctuating conformation. Dill and Shortle (1991) have performed extensive research on the denatured state of the proteins in the presence of strong denaturants such as 6 M guanidinium chloride (GdmCl) or 8 M urea. They found that unfolded proteins have the average hydrodynamic properties expected for random coil polypeptide copolymers. The energetics of the intramolecular interactions in the random coil are exactly balanced by interactions with the solvent (Dill and Shortle, 1991).

1.3.3 "Molten Globule," a Compact Intermediate State (M)

Under certain conditions of pH, salt, and temperature, a variety of proteins have been observed to form stable conformations that are neither fully folded nor fully unfolded. The occurrence of conformations with nativelylike secondary structure and a liquidlike interior is known as the molten globule state. The molten globule state is a compact denatured state with a significant amount of nativelylike secondary structure, but a largely disordered tertiary structure. It has been proposed to be a major intermediate along the folding pathway for many proteins (Kuwajima, 1989; Ptitsyn, 1992; Baldwin, 1993; Jennings & Wright, 1993). Studies of thermally unfolded ribonuclease A at low pH (1.5-3.8) using CD and pulsed H/D exchange (Baldwin, 1993), and those using small-angle X-ray scattering and Fourier transform infrared spectroscopy (Sosnick and Trewella, 1992) show that the partially denatured protein has some properties of the molten

globule state. Also, Fink et al. (1994) have investigated the acid-induced unfolding of 20 small monomeric proteins as a function of ionic strength. With an increase in ionic strength at low pH, some proteins initially unfold and then refold into a compact molten globule conformation known as the acid denatured or A state. The A state adheres to the characteristics of a molten globule on the basis of compactness and amount of the secondary structure.

Characteristics of the molten globule state as a hypothetical common intermediate are described by Ptitsyn (1992): (1) The molten globule state is almost as compact as the native protein; (2) there is a high content of secondary structure in the molten globule state compared to the fully unfolded state; (3) there is clear evidence that the molten globule has a relatively dense core; (4) in the molten globule state, the approximately 50% increase in a protein's volume suggests that water can penetrate inside; (5) the local environment of the aromatic side chains in the molten globule state is much more flexible than in the native state.

1.4 Dominant Forces in Protein Stability

The free energy of stabilization of globular proteins in the folded state under physiological conditions is found to be marginal. Values (ΔG_{unfold}) commonly determined for globular proteins are largely independent of the mode of denaturation.

$$\Delta G_{\text{unfold}} = G_{\text{denatured}} - G_{\text{native}} \quad (1)$$

They are typically about 20-70 kcal/mole. This is less than $1/10kT$ per residue, where k is Boltzmann's constant and T is temperature (Seckler and Jaenicke, 1992). As a result, many types of molecular interactions contribute to the stability of proteins. Forces involved in protein folding are: electrostatic interactions, hydrogen bonding and van der Waals interactions, and hydrophobic interactions.

1.4.1 Electrostatic Interactions

The dominant forces that stabilize proteins were first assumed to be electrostatic in nature, because acids and bases were among the earliest known denaturants. The electrostatic interactions are a consequence of the charge distribution on the protein. Therefore, they differ in the native and unfolded states of the protein. Depending on the positive or negative charge of the side chain in the protein, the electrostatic interactions can be either attractive or repulsive.

There are two different ways in which electrostatic interactions can affect protein stability: (1) "Classical" electrostatic repulsions, and (2) "Specific" electrostatic attractions. The overall contribution of electrostatic interactions to the stability of a protein is determined by the difference between the sum of the attractive and repulsive interactions in the protein.

"Classical" electrostatic interactions are the nonspecific repulsions that arise when a protein is highly charged. Proteins are ampholytes that contain both

acidic and basic side chains. At one particular pH, the net charge in the protein is zero. This is called the isoelectric point (pI). At pH values above or below pI, the overall charge on the protein will be either negative or positive charges respectively. The greater the difference between the pH and pI, the larger the total charge will be. At extremes of pH, the repulsive forces may lead to denature the protein. Pace et al. (1990) have investigated the pH dependence of urea and guanidinium chloride denaturation of RNase A and RNase T1. Their results show that proteins are most stable at pH values near their pI.

“Specific” electrostatic attractions can help stabilize protein structures through ion pairs (salt bridges). Ion pairing occurs when these opposite charged amino acid side chains are in close spatial proximity. Most charged groups are exposed to the aqueous solvent. On the average, only one ion pair per 150 amino acid residues of a protein is buried within the interior core (Jaenicke, 1991). Thus, only surface ion pairs are expected to be involved in stabilization. This agrees with studies of X-ray crystal structures of known proteins showing that ion pairing is common on the surfaces of proteins (Anderson et al., 1990).

The role of electrostatic interaction in protein stability has been argued extensively. In particular, several researchers suggest that ion pairs are strong, but not the dominant contributor to protein stability (Hollecker and Creighton, 1982; Pace and Grimsley, 1988; Stigter and Dill, 1990; Jaenicke, 1991). However, recent studies by Dym et al. (1995) suggest that ion pairs are abundant and stronger in

the proteins of thermophilic organisms, and are one major contributor to these protein thermostability.

Several lines of evidence indicate that electrostatic interactions contribute significantly to protein stability. A recent polyelectrolyte model of proteins (Stigter and Dill, 1990) shows, by classical effect alone, that increasing salt will reduce the electrostatic free energy of the unfolded state of myoglobin more than the folded state. The increased salt shields the charge repulsions in the unfolded molecules more effectively than in the folded molecules at low pH. They suggest that this is due to better penetration of the salt solution into unfolded molecules. Further, Pace et al. in 1990 suggested that electrostatic interactions among the charged groups make a contribution to the conformational stability of these proteins. However, it is difficult to discriminate between classical and ion pairing electrostatic interactions towards protein stability (Jaenicke, 1991; Seckler and Jaenicke, 1992).

1.4.2 Hydrogen Bonding and van der Waals Interactions

A hydrogen bond occurs when a hydrogen atom is effectively shared between two electronegative atoms. Hydrogen bonds are a major force involved in the formation and stabilization of secondary structures such as α -helix and β -sheet. These bonds involve the interaction of the amino hydrogen and carbonyl oxygen of the polypeptide backbone. Hydrogen bonds can also be formed between

hydrogen donors and acceptors on the side chains of some amino acids (Figure 1). These play a significant role in conferring the structure specificity to the native state of the protein. The intrinsic energy of hydrogen bonds is usually -2 to -8 kcal/mole and the strength increases as the distance between the hydrogen bond donor and acceptor decreases.

The van der Waals forces are the noncovalent associations between electrically neutral molecules. The van der Waals attractions in proteins arise from interactions between tightly packed hydrocarbon side chains in the hydrophobic core of the protein. There are different types of van der Waals forces, such as fixed dipole and dipole-induced interactions. The amino acids that comprise proteins are dipolar. Especially, the carbonyl and amide groups of the peptide backbone have fixed dipole moments. Therefore, fixed dipoles are important structural determinants in proteins. These interactions are different and much weaker than the charge-charge interactions of ion pairs. A fixed dipole can also induce a dipole moment on a neighboring group and, in doing so, form an attractive interaction. Such dipole-induced interactions are generally much weaker than dipole-dipole interactions.

Solvent denaturation studies conducted by Singer (1962) with nonaqueous solvents suggest that hydrogen bonding is not the dominant force in protein folding. In general, the peptide carbonyl group is a strong hydrogen acceptor and the peptide amino group is a weak hydrogen donor. Singer pointed out that

Hydrogen donor
group of Trp

Hydrogen donor
group of Arg

Asn or Gln

Asp or Glu

Figure 1: Hydrogen bond donors and acceptors for amino acid side chains found in proteins.

solvents useful for competing with the peptide hydrogen bond must be stronger hydrogen donors than the amide group, and stronger hydrogen acceptors than the carbonyl group. For example, dioxane is only a weak hydrogen bond acceptor. Therefore, dioxane should not denature proteins if hydrogen bonding is the dominant force in protein stability. However, dioxane has been shown to denature proteins (Singer, 1962).

According to Creighton, “within the polar aqueous environment, noncovalent interactions, hydrogen bonds or van der Waals forces, are not expected to be especially favorable energetically, because similar interactions will occur between the polypeptide and the water surrounding it” (Creighton and Goldenberg, 1984). However, recent studies have shown that hydrogen bonding plays a significant role in protein stability. The calorimetric studies reported by Makhatadze and Privalov (1992) indicate that hydrogen bonding of urea or guanidinium ion to the amide backbone of proteins is a major contributor to the denaturation process. The work of Chen et al. (1993) has shown that a single hydrogen bond can make a significant contribution to protein stability.

1.4.3 Hydrophobic Interactions

More than 30 years after Kauzmann’s (1959) insightful hypothesis, there is now strong accumulated evidence that hydrophobicity is the dominant force that directs protein folding. It now appears that it is the absence of interactions

between the nonpolar molecules and water, rather than a favorable interactions between the nonpolar groups themselves, that is a major factor in protein stability. This has become known as the “hydrophobic effect.” The hydrophobic effect is derived from the special properties of water. When nonpolar groups of the protein are introduced into water, there are both enthalpic and entropic changes in the system. In the unfolded state, both hydrophilic and hydrophobic side chains are exposed to water. The exposure of hydrophobic residues results in the ordering of water which reduces the entropy of this local region and is energetically unfavorable. Therefore, the hydrophobic effect will drive nonpolar residues of the polypeptide chain in water to form a hydrophobic core which serves to maximize the solvent entropy. Compaction to the native state leads to the burial of hydrophobic residues which decreases their total surface area in contact with water and allows for an increase in the entropy of water. For this reason, the hydrophobic effect not only stabilizes the protein structure, but also drives proteins to form compact structure.

The solvent-driven compaction is opposed by the unfavorable change in the solute entropy which arises due to the localization of hydrophobic residues in the compact and hydrophobic protein core. Chan and Dill (1990) have examined the compaction of the polypeptides through computer modeling using a “lattice model.” They observed that compaction of polypeptides into regularly folded patterns yielded a higher solute entropy than if the polypeptides were folded

randomly. This means that the solute entropy decrease is less for ordered compaction and will be the preferred mode for compaction. Altogether, hydrophobic effect leads to compact conformations with nonpolar cores, but it is the steric constraints in compact chains that are largely responsible for the native state of proteins.

There is a considerable amount of evidence which continues to support the view that hydrophobic interaction is the dominant force of protein folding. Bowie et al. (1990) suggest that the hydrophobicities of residues in the core of the protein appear to be more strongly conserved and are correlated with structure to a greater degree than other types of interactions.

It seems reasonable to conclude that most of the interactions within the folded protein are more energetically favored under physiological conditions than corresponding interactions of the unfolded state. They should, therefore, contribute to the net stability of folded state and the hydrophobic interaction is probably the major stabilizing factor (Creighton, 1990).

1.5 Models of Protein Folding

The process of protein folding has been examined for nearly 60 years. By 1925, the denaturation process was considered to involve either hydrolysis of the protein peptide bond or the dehydration of the protein. Historically, Wu concluded that protein denaturation was a process involving the unraveling of the polypeptide

chain (Wu, 1929). He also proposed that the structures of native proteins involve regularly repeated patterns of folded polypeptide chain arranged into a three-dimensional network, somewhat resembling a crystal and held together by noncovalent linkages. Models describing the protein folding pathway were developed further until the remarkable discovery of Anfinsen in the late 1950's. He demonstrated that the amino sequence of a protein, a one-dimensional trait, was fully sufficient to specify the molecules' ultimate three-dimensional shape and biological activity.

In the last few decades, technological advances have made it possible to obtain the precise knowledge of the three-dimensional structure of proteins. In particular, X-ray crystallographic and NMR studies have provided data necessary for understanding how the architecture of a protein molecule is built from the extended polypeptide chain. Unfortunately, these techniques are only useful for determining the native structure of proteins and cannot be readily applied to nonnative species. For this reason, the pathway of protein folding is still not clear. However, several models have been proposed to explain the protein folding process.

1.5.1 Random Search Model

Levinthal (1968) first considered the possibility that proteins might fold by a purely random search of all possible conformations. However, assuming the

polymer segments move at the maximum rate of diffusion, the time required for folding a protein with 100 residues was calculated to be 10^{50} years (Schmid, 1993). Thus, even for a small protein, it would take considerably longer than the age of the universe to form its native structure. However, it is possible that a biased random search could occur in the time frame required for protein folding in vivo. Levitt (1975) showed, by means of computer-simulated folding for a lattice model, that the number of possible chain conformations is drastically reduced if only self-avoiding conformations are allowed. This work suggests that a biased random search may play an important role in the early stages of folding (Levitt, 1975).

1.5.2 Nucleation Rapid Growth Model

The nucleation rapid growth model was proposed by Wetlaufer (1973). According to this model (Figure 2), a nucleation center is formed in the early stage of a folding pathway through random conformational fluctuations of the unfolded protein. This center, comprised of a single intramolecular interaction, serves as a template upon which folding proceeds rapidly to completion. This nucleation center is unstable by itself and breaks down if it is not stabilized by further folding.

Formation of nucleation center is the rate-limiting step in this model. Therefore, folding is observed as one single step with no well populated intermediates in folding pathway. The nucleation rapid growth model does not

Figure 2: Nucleation rapid growth model. Schematic representation of the native (N), intermediate (I) and unfolded (U) states. The nucleation center is indicated by the arrow.

explain a significant amount of experimental data. In particular, this model does not account for the existence of intermediates observed in the folding of a number of proteins. For this reason, it may not be generally applicable to the folding of all proteins.

1.5.3 Diffusion-Collision Model

The diffusion-collision model views the protein as composed of several parts called microdomains (Karplus and Weaver, 1976, 1979). These microdomains may be portions of incipient secondary structures (α -helices or β -sheets) or hydrophobic clusters. The microdomains move diffusively, and microdomain-microdomain collisions take place. Collisions can lead to coalescence into multimicrodomain intermediates. Then, folding proceeds as a series of coalescence steps that might follow either a sequential (single folding pathway) or parallel folding pathway (multiple pathway). Overall folding would lead to the native conformation of protein. A pictorial representation is shown in Figure 3. Karplus and Weaver (1994) calculated the rate for a protein to fold by a diffusion-collision mechanism and found that the diffusive collision to form the microdomains would be the rate-limiting step.

Kim and Baldwin (1982) studied the kinetics of folding of RNase A as a function of solvent viscosity. It was found that the overall folding rate was independent of solvent viscosity. They concluded that diffusion is not rate-limiting

Figure 3: Diffusion-collision model. Pictorial representation of kinetic event involved in the diffusion-collision model of small globular proteins and protein domains. Microdomains (A-G) indicated by dashed outlines are individually unstable. Stable coalescent intermediates indicated by solid outlines are held together by hydrophobic interactions. Reproduced with permission from Karplus and Weaver, 1994, copyright 1994, by Cambridge University Press.

for the folding of this protein. In contrast, Karplus and Weaver (1994) examined model polymers and found that the diffusion of one segment to another decreased with increasing solvent viscosity, indicating that diffusion is rate limiting for the folding of these polymers.

1.5.4 Molten Globule Model

Kuwajima (1989) proposed the molten globule model to explain early intermediates observed in kinetic studies of protein folding. In this model, the intermediate is described as a compact globular molecule with natively like secondary structure, but unfolded tertiary structure. The hypothesis upon which this model is based implies that the “frame” of the native protein is essentially preserved in the molten globule state, while other parts of the molecule can be more or less unfolded (Figure 4). The frame includes the central parts of the main α -helices and β -sheets and those parts of regions carry nonpolar side chains contributing to the hydrophobic core. It has been proposed that the molten globule state forms early in folding and the rate determining step involves later structural changes after the accumulation of the molten globule state.

1.5.5 Hydrophobic Zipper Model

Dill et al. (1993) have presented the hydrophobic zipper model of protein folding cooperativity. The idea behind the hydrophobic zipper model is that monomeric hydrophobic residues pair together one after another, like the zipping of a zipper (Figure 5). Any two hydrophobic residues near enough in the primary

Figure 4: Molten globule model. Schematic representation of the native and molten globule states of the protein molecule. The cylinders represent α -helical structures and nonpolar side chains are represented by the hatched patterns. Reproduced with permission from Creighton, 1992 copyright by W. H. Freeman and Company, 1992.

Figure 5: Hydrophobic zipper model. Pictorial representation of the hydrophobic zipper model. The closest hydrophobic (H) residues (solid dots) in sequence pair first, constrain the chain and bring other H monomers into proximity. As H contacts form, to develop a core, helices and sheets zip up. Reproduced with permission from Dill et al., 1993 copyright National Academy of Sciences USA

sequence can readily find each other through a small conformational search with little loss of conformational entropy. Contact between the hydrophobic pair now serves as a constraint which forces other hydrophobic monomers into spatial proximity and additional pairing occurs. The process continues and the hydrophobic pair contacts form a core and “zips up” the next contacts. The hydrophobic zipper-heteropolymer collapse is a highly cooperative process driven by nonlocal interactions. Furthermore, the collapse itself results in the formation of sheets and helices.

This model was tested through computer modeling of crambin with 46 residues, and bovine pancreatic trypsin inhibitor (BPTI) with 58 residues. The best structures predicted by this model have energies within about 1.5 kcal/mole of actual native structures (Dill et al., 1993). Although the hydrophobic zipper search strategy shows promise, extensive research is necessary to confirm this model.

1.5.6 Sequential Model

The sequential model is currently the most popular. According to Kim and Baldwin (1982), the sequential folding pathway occurs in a unique and definite sequence of steps. The simple model is as follows:

where N and U represent the native and unfolded states, respectively, and I_i are the various intermediate structures.

In this model, protein folding consists of at least three stages: (1) the formation of fluctuating secondary structure in an unfolded chain, (2) the merging of preexisting blocks with a secondary structure to an intermediate globular structure, and (3) the adjustment of this intermediate structure to the final specific native tertiary structure. The essential feature of this model is that different states are stabilized by different interactions: (1) secondary structure, by peptide hydrogen bonds, (2) intermediate globular structure, by hydrophobic interactions, and (3) specific tertiary structure, by van der Waals interactions (Ptitsyn, 1987).

The sequential folding model assumes the existence of specific, well populated intermediates and their formation and decomposition occurs in a sequential manner. Experiments with RNase A performed by several laboratories (Schmid and Baldwin, 1979; Kim and Baldwin, 1990; Biringer and Fink, 1988) support the sequential model.

In conclusion, experimental data provide some support for all models except the random search model for protein folding, but also provide contradictions. Given the complex nature of protein folding, the processes that lead to the native protein from a random polypeptide chain most likely involve certain elements of the proposed models.

1.6 General Approach for Protein Folding Study

The general approach for studying protein folding has been to study either the equilibrium transition or the kinetics of unfolding or folding.

1.6.1 Equilibrium Studies

Native proteins can be unfolded by incremental changes in temperature, pH, or by adding chemical denaturants under the equilibrium condition. The equilibrium is shifted to favor the unfolded state at extremes of pH, high concentration of denaturants or high temperature. Many small single domain proteins unfold reversibly and in a cooperative manner. The two-state nature of protein folding transitions indicates that folding is a cooperative process which in turn indicates that intermediate conformations are not highly populated (Creighton, 1990). Thus, such a transition involves only native (N) and unfolded (U) molecules. The equilibrium transition is described by:

At any point in the transition, the relative population of native and unfolded molecules is related by the equilibrium expression:

$$K_{eq} = [U] / [N] \quad (4)$$

Also, the fraction of unfolded molecules (F_u) for a two-state transition is given by:

$$F_u = [U] / ([N] + [U]) \quad (5)$$

The free energy difference (ΔG) between the native and unfolded states, is defined by:

$$\Delta G = -RT \ln K_{eq} \quad (6)$$

where R is ideal gas constant and T is the absolute temperature.

Cooperativity of denaturant-induced unfolding is often inferred if it occurs over a limited range of denaturant concentration to give a sigmoidal unfolding curve. Figure 6 shows GdmCl denaturation curve which is a typical two-state folding transition. The solid line represents the fraction of unfolded molecules (F_u) that was observed experimentally. Within the transition region, 2.8 to 3.2 M GdmCl, the free energy difference (ΔG) between the native and unfolded states for this protein is usually observed to be linearly independent upon the concentration of GdmCl. The net stability of the folded state outside the transition region must be determined by extrapolation. Thus, extrapolation of the free energy values to zero denaturant concentration (dash line in Figure 6) gives the ΔG for the process in the absence of denaturant (Creighton, 1990) which is a good measure of the overall stability of the protein.

Unfolding curves obtained using different structural probes under the same denaturation conditions should be coincident if the process is two-state transition. Noncoincident curves indicate that the unfolding process is multi-state, and that

Figure 6: Typical denaturation curve. Solid line gives data as the fraction of unfolded molecules (F_u) versus guanidinium chloride (GdmCl) concentration. The dotted line gives the change in free energy (ΔG) and the log of the equilibrium constant (K_{eq}) vs. [GdmCl].

intermediates are present at equilibrium. Data for such transitions are typically treated as the sum of individual two-state processes.

For a noncooperative transition, the two-state approximation is not valid. In this situation, one or more partially folded intermediate states (I_i) may be populated on the pathway. For the simplest model, only one intermediate presents in the unfolding process and can be treated by the following:

The overall equilibrium constant will be a product of the individual equilibria as given by:

$$K_{eq} = K_1 K_2 \quad (8)$$

where $K_1 = [I] / [N]$, and $K_2 = [U] / [I]$, respectively. In this case, the apparent free energy is the sum of the individual free energies. For this reason, the plot of the apparent free energy against the concentration of denaturant will be nonlinear for multiple states.

1.6.2 Kinetic Studies

The kinetics of protein refolding are examined by first unfolding the protein with heat, denaturant, or other means and then rapidly changing the experimental conditions to favor the native state. The folding process is followed by monitoring changes in the values for structural probes, usually spectroscopic,

as a function of time. Protein unfolding kinetics are measured in a reverse fashion; the native protein is introduced to conditions which favor the unfolded state.

In the simplest case of folding or unfolding kinetics, a single kinetic phase is observed. Such results indicate that partially folded intermediates do not accumulate to any substantial levels (Schmid, 1993). Multiphasic kinetics may represent the sum of two or more single exponential processes, a series of different processes in a sequential pathway, or combination of both.

1.7 Measurement of Protein Folding

Numerous methodologies have been employed to examine the process of protein folding and unfolding. Chief among them are absorbance and fluorescence spectroscopy which serve to measure the gross three-dimensional structure of the protein, and circular dichroism which serves to measure the secondary structure. Each of these methods monitors different properties of the protein during the folding process. Although these methods have proven invaluable in the study of protein folding, the information that they individually give does not provide a complete picture of the process. However, a more concise picture of the folding process can be obtained by comparing data from a variety of techniques that are sensitive to different changes in the protein structure.

1.7.1 Absorbance and Fluorescence Spectroscopy

Both UV absorbance and fluorescence spectroscopy provide a measure of the tertiary structure of a protein. The signals obtained from these techniques provide a measurement of the microsolvent environment about the aromatic species. For this reason, the UV absorption maxima of proteins undergo wavelength shifts and intensity changes as the secondary and tertiary structures of molecules are disrupted. This phenomena can be used to monitor the unfolding process. Absorbance of UV light by peptide bonds and nonaromatic residues are not very sensitive to changes in conformation and environment whereas aromatic residues such as phenylalanine, tyrosine and tryptophan are.

In case of RNase A, there are six tyrosine residues, but no tryptophan. Absorbance properties of the molecule are dependent on the microsolvent environment of the chromophores. For aromatic side chains, the higher the polarizability of the environment, the lower energy of the electronic transition. and a corresponding lower wavelength maximum for the absorbance band. In the native state of RNase A, there are three tyrosines exposed to the solvent and three buried inside the hydrophobic core of the protein. The absorbance spectrum for residues buried in the native protein will occur at longer wavelengths than when they are exposed to the solvent in the unfolded state as a result of the difference in polarizability between the different environments. Since the interior of a protein is

considerably more hydrophobic and thus more polarizable than the bulk water solvent, denaturation of RNase A is accompanied by a shorter wavelength shift of the absorbance band (hypochromic shift) and a decrease in absorbance.

The aromatic amino acids act as intrinsic fluorescent probes in proteins. The fluorescence of these side chains is lower when the protein is folded than when it is unfolded. This is due to the fact that aromatic side chains are effectively quenched when buried in the native structure and quenching is less effective when the protein is unfolded and the residues are exposed to the solvent. Further, this technique is more sensitive to changes in the environment than absorbance due to the longer lifetimes of the excited states. The longer lifetimes allow for the environment to significantly alter the electron distribution of the excited state, and such alternations have a profound effect on the resulting signal. Consequently, intrinsic fluorescence can serve to monitor more subtle changes in structure than by absorbance measurements (Stryer, 1968).

1.7.2 Circular Dichroism

Circular dichroism (CD) is a most sensitive probe of the polypeptide backbone conformation of globular proteins. CD is a measure of the difference in absorption of left- and right-handed circularly polarized light. In the far-UV region (below 250 nm), the signal results from the absorbance by the polypeptide backbone which is sensitive to conformation. Secondary structures such as α -

helix, β -sheet, turn and random coil are optically active and each of these structures has a characteristic CD band between 178 and 240 nm (Johnson, 1985).

The ellipticity at 220 nm serves as a good measure of the α -helix content since α -helix is the major contributor at this wavelength. Previous studies have shown that α -helix does not contribute to the ellipticity at 201 nm and all other secondary structures contribute significantly. Therefore, ellipticity at 201 nm can serve to monitor secondary structures other than α -helix.

1.7.3 ANS Binding

Soluble proteins have few hydrophobic regions on their surface. In the early stage of unfolding, however, solvent accessible hydrophobic pockets are formed. Hydrophobic fluorescent dyes have been shown to have ability to bind these exposed hydrophobic regions. Binding typically results in an increase in the quantum yield of the dye, as the microsolvent environment within the pocket is considerably more hydrophobic than in the bulk solvent. In the latter stages of unfolding, the pockets are dismantled. Although the dye might still bind to exposed hydrophobic regions, the average microsolvent environment will be more polar resulting in a decrease in the overall quantum yield. Hence, changes in the dye's fluorescence quantum yield can serve to measure these alternations in protein structure.

1-anilinonaphthalene-8-sulfonate (ANS) is a typical dye used in such studies. ANS is an anionic amphiphile and is fluorescent (fluorescence absorption/emission at 370/482 nm). Its structure is shown in Figure 7.

Figure 7: ANS structure

1.7.4 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy has proven to be a powerful tool for monitoring protein folding as well as for elucidating protein structure. Blum et al. (1978) and Biringer and Fink (1982) used the C-2 protons of the four histidine (12, 48, 105, 119) residues of RNase A to monitor protein folding. When partially folded intermediates were present during folding, new transient resonances were observed with chemical shifts that were different from both native and unfolded protein. By following the time-dependent changes in the magnitude of these resonances, Biringer and Fink confirmed the existence of partially folded intermediates.

1.8 RNase A as a Model of Protein Folding

The protein chosen for this study was bovine pancreatic ribonuclease A (RNase A). RNase A is a small, globular protein that catalyzes the hydrolysis of ribonucleic acid. The protein has been thoroughly characterized and consists of a single polypeptide chain of 124 amino acids with four disulfide linkages, four prolines, and six tyrosines. Three of the tyrosine residues (73, 76, 115) are considered to be “exposed” to the solvent in the native structure and the other three tyrosines (25, 92, 97) are located in the hydrophobic core of the protein. This enzyme is known to undergo reversible unfolding without loss of catalytic activity.

This particular protein offers a number of advantages: (1) The crystal structure is well known; (2) its folding is characterized by multiple intermediate states; (3) its solubility in aqueous or aqueous-organic solvents is very high; (4) it has a low tendency to aggregate; (5) its unfolding and subsequent refolding is reversible; (6) it has been well studied by our group and others.

1.9 Purpose and Significance of Project

The relative importance of the various factors that contribute to the stability of proteins has been the subject of controversy for many years. It is apparent from the vast amount of experimental work that hydrogen bonding and the hydrophobic effect are the major contributors to protein stabilization. One way

to investigate the hydrophobic contribution is to examine the thermal or chemical denaturation of a protein in presence of hydrophobic solvents. In order to examine hydrogen bonding contribution, denaturation in the presence of denaturants that act as hydrogen donors or acceptors is examined.

It is well established that monohydric alcohols destabilize the tertiary structure of proteins yet apparently serve to stabilize the secondary structure, in particular, α -helix. The decrease in the stability of the tertiary structure by the action of monohydric alcohols is due to the unfavorable interaction between the solvent and the hydrophilic protein surface and the favorable interaction with buried hydrophobic side chains. On the other hand, partially folded intermediates in protein folding are expected to have more solvent-exposed nonpolar surface than the native state. For this reason they will be more stable in the hydrophobic cosolvent than the native state due to the more favorable interaction between hydrophobic surface area and less polar cosolvent.

The thermal denaturation of proteins in the presence of alcohols has been observed to be complex in the presence of high concentrations of alcohols. For example, the proton NMR data presented by Biringer and Fink (1982) show that the number of partially folded states observed in the thermal denaturation of RNase A increases with increasing methanol concentration. Two reports by Fink and coworkers (Fink and Painter, 1987; Lustig and Fink, 1992) show that the fluorescence- and CD-monitored thermal denaturation transitions for RNase A are

not coincident at high concentrations of methanol. Lustig and Fink (1992) have shown that the loss of the tertiary structure occurs at lower temperatures than the loss of the secondary structure in presence of high concentration of methanol (50% v/v). Their data is consistent with the formation of an intermediate that lacks the native tertiary structure, but retains some native secondary structure, in particular, α -helix. This type of compact intermediate state bears some resemblance to the state known as the molten globule state.

In order to examine the universal nature of uncoupling of the secondary structure from the tertiary structure by methanol, Biringer et al. (unpublished data) examined the effect of methanol on the urea-mediated denaturation of RNase A. The results obtained from ANS binding experiments were particularly interesting. The ANS binding data for RNase A denatured by urea in aqueous and particular aqueous-methanol solvent systems are given in Figure 8. In aqueous and 25% (v/v) methanol, the data shows an enhanced ANS binding with increasing urea at low concentrations of urea followed by a complete loss of signal at higher concentrations of urea. Such results are consistent with the stabilization of a molten globule state at low concentrations of urea (a fluorescence maximum near 2.5 M urea). At higher concentrations of methanol, the data becomes bimodal which is consistent with the formation of two such states. Clearly, methanol is stabilizing and populating a second molten globule state.

Figure 8: Urea denaturation of RNase A in the presence of methanol at pH* 4 and 17 °C as monitored by ANS binding. Data is presented as the relative fluorescence emission of ANS (400:470 nm). Protein and ANS concentrations were 3.6 and 72 μ M respectively. Circle gives the concentration of methanol 50% (v.v). Taken from Biringer's unpublished data.

Characterization of these molten globule states by differential scanning calorimetry (DSC) was conducted by Tiktopulo et al. (unpublished data). The results are given in Table 1. Two parameters can be gleaned from the data, the enthalpy associated with any observed event and the T_m , the temperature of maximal heat absorption. The latter is equivalent to the temperature required for 50% of the observed event to be complete which is directly comparable to thermal transition midpoint obtained with other techniques. It is clear from the data that the denaturation of RNase A appears to be two states unless both methanol and low concentrations (0.5-1.0 M) of urea are present. Such results indicate that only the molten globule state that is stabilized by methanol is associated with a major structural event.

The goal of this project was to take these studies to the next logical step. That is, to characterize the structure of the molten globule states in a more precise manner. For this purpose, the thermal denaturation of RNase A in 50% methanol and various concentrations of urea were examined at pH* 3.0 by fluorescence and CD.

Table 1: Thermodynamic Parameters for Thermal Denaturation of RNase A by Differential Scanning Calorimetry

0.1M KCl pH* 3.0	T _{m1} °C	ΔH ₁ kcal/mole	T _{m2} °C	ΔH ₂ kcal/mole	ΔH _{tot}
0% MeOH	44.0	78.5	----	----	78.5
50% MeOH	26.8	67.0	----	----	67.0
pH* 3.0, 50% MeOH, 0.1M KCl					
+0.5M urea	25.6	56.0	34.2	37.0	93.0
+1.0M urea	22.2	60.0	32.6	30.0	90.0
pH* 3.0, 50% MeOH					
+0.5M urea	26.2	44.0	30.5	35.0	79.0
+1.0M urea	16.9	53.0	31.1	33.0	86.0
+1.5M urea	14.2	29.5	----	----	29.5
pH* 3.6, 50% MeOH, 0.1M KCl					
+1.5M urea	28.4	92.0	----	----	92.0

^a T_m values and ΔH values were taken from Tiktopulo E. et al. (unpublished data).

^b pH*, MeOH, T_m and ΔH stand for protonic activity, methanol, transition midpoint in thermal denaturation and enthalpy respectively.

Chapter 2: MATERIALS AND METHODS

2.1 Materials

Bovine pancreatic ribonuclease A was purchased from Sigma Chemical Co. (St. Louis, MO). Ultrapure urea and guanidinium chloride were purchased from Schwartz-Mann Biotech. HPLC grade methanol was purchased from Fisher Scientific.

2.2 Methods

2.2.1 Purification of Ribonuclease A

RNase A was further purified on a SPC-25 (Pharmacia™) ion-exchange column (1.5 × 25 cm) using 0.12 M phosphate buffer at pH 6.5 as the eluent (Biringer and Fink, 1988). In a typical purification, 150 mg of RNase A was dissolved in 2-3 mL of the eluting buffer and loaded on to a preequilibrated column. Then, the sample was eluted at a 0.5 mL/min flow rate at room temperature. This procedure facilitates the separation of RNase S and aggregated material from RNase A. The pure monomeric fraction of RNase A was collected and dialyzed for 2 hours against 20 liters of deionized-distilled water in the cold room. The dialyzed solution was refreshed with deionized-distilled water and left

overnight. After dialysis, the solution contains less than one ppt of phosphate (Biringer and Fink, 1982). The dialyzed protein was frozen and lyophilized at -20 °C.

2.2.2 Preparation of Protein Stock Solutions

Stock RNase A solutions were prepared by dissolving the protein in the appropriate buffer to give a final concentration of 5 mg/mL. The buffer solutions were 50% aqueous-methanol in 0.1 M KCl at pH* 3.0 in absence or presence of various concentrations of urea. The protein solution was mixed slowly by magnetic stirring to prevent foaming. A 0.45 µm teflon membrane (Millipore™) was used to filter dust and other particles from the protein solution. The concentration of the protein stock solution was determined spectrophotometrically. A 50 µL aliquot of the protein stock solution was added to 800 µL of aqueous buffer (0.1 M acetate) at pH 6 in a 1 mL spectrophotometer cell. After stirring to homogeneity with a hand held motorized stirrer, the absorbance was measured at 278 nm using 0.1 M acetate at pH 6.0 as the blank. The concentration of the protein stock solution was calculated from Beer's law with these parameters: MW = 13,800, $\epsilon_{278} = 9800$ AU/M cm, and $b = 1$ cm (Sage and Singer, 1962). The stock solution was stored in the freezer and used within two weeks.

2.2.3 Preparation of 50% Methanol with and without Urea

The 50% aqueous-methanol solvents were made on a volume to volume basis. In all experiments, deionized-distilled water and HPLC grade methanol were used. Aqueous solvents were prepared by mixing equal volumes of 0.2 M KCl and 0.1 M formate buffer to give the final concentration of 0.1 M KCl and 0.05 M formate and the pH adjusted to 3.0.

For 50% aqueous-methanol solvent without urea, equal volumes of aqueous buffer and methanol were mixed and then carefully adjusted to pH* (apparent pH) 3.0. The pH* of cryosolvent solutions was determined at 22 °C using a glass combination electrode. The solutions were filtered through a 0.45 µM membrane. For 50% aqueous-methanol solvent with urea, the required amount of ultrapure urea was added into the required volume of aqueous-methanol solvent. Three urea concentrations were prepared, 0.5 M, 1.0 M and 1.5 M. The appropriate volume of concentrated protein stock solution was added to the appropriate solutions and mixed by vigorous vortex mixing. The final concentration of the protein after addition to the particular 50% aqueous-methanol solution with or without urea was 20 µM. The solutions were allowed to equilibrate at room temperature for 30 minutes prior to taking any measurements. This procedure was followed to allow the protein to unfold to an equilibrium population of molecules.

2.2.4 The Temperature Control of Thermal Unfolding Transitions

The experimental temperatures were maintained by using cells jacketed in insulated brass blocks and thermostated using circulating ethylene glycol-water mixtures from a Neslab™ RTE 110 constant temperature bath. A Neslab™ linear temperature programmer was used to control and ramp the temperature of the RTE 110 bath. Heating and cooling rates were set to 0.3 °C per minute. Cooling ramps of 0.3 °C per minute required that a heat exchanger from an additional RTE 110 bath, set at subzero temperature, be placed directly into the solvent reservoir of the ramping bath. Experimental temperatures were continuously monitored with an Omega™ recording thermometer. The temperature probe itself was placed on the side of the cuvet. The chart recording was calibrated by comparison with the output from an Omega™ HH-71 digital thermometer attached to the same temperature probe. The temperature range examined for aqueous buffer and aqueous-methanol buffer was accomplished at 17-65 °C and -10-70 °C, respectively. The temperature limit for 50% aqueous-methanol was limited to a maximum of 75 °C, since this temperature is close to the boiling point of the solvent. At subambient temperatures a nitrogen purge was applied to optical faces of the cell to prevent the accumulation of moisture. Cells were sealed with teflon stoppers to prevent evaporation.

2.2.5 Fluorescence-Monitored Thermal Unfolding Transitions

All fluorescence measurements were performed with a Spex Fluorolog™ Spectrofluorometer interfaced to a computer for data acquisition and analysis. All fluorescence measurements were made with an excitation wavelength of 280 nm and emission wavelength of 305 nm. The computer mode was set to the kinetic mode (time base scan). The total observation was set to 12,000 seconds, time increment between measurement was 30 or 60 seconds, and integration time was 1 second. Fluorescence versus time data was compared to the temperature recorder output to convert it to fluorescence versus temperature data. Thermal denaturation was completed reversibly for the same protein solution at all of the conditions reported here.

2.2.6 CD-Monitored Thermal Unfolding Transitions

The far UV-CD measurements were carried out on a Cary™ 61 Spectropolarimeter. The CD instrument was continually purged with dry nitrogen to prevent the formation of ozone. All measurements were made with a 5.00 mm pathlength cylindrical CD cell. The parameters were set as followings: chart speed was 1 nm/division, degree scale was 0.1 or 0.2, period was 10, and scan rate was 1 nm/sec. The spectra were scanned from 260 nm to 210 nm. Each spectrum was taken after temperature equilibration for 8-10 minutes and repeated twice. The reversibility of thermal folding also was examined using the same sample.

The calibration of the CD instrument was routinely checked using a 0.2% (w/v) 10-camphorsulfonic acid (CSA). The CSA standard was prepared in deionized-distilled water and the concentration determined by UV absorbance at 286 nm ($\epsilon = 39.5$ AU/M cm). The CSA standard solution was placed in a 1 mm pathlength cell and scanned at the rate of 0.2 nm/sec using 0.2 degree scale at room temperature. The baseline was adjusted by using deionized-distilled water as the blank. Both sample and blank were scanned from 350 nm to 180 nm. The spectrum shows two major peaks at 290 nm and 190 nm. The intensity at 290 nm ($\epsilon = +2.36$ AU/M cm) and 190 nm ($\epsilon = -4.80$ AU/M cm) were used to confirm the calibration.

2.2.7 Data Analysis

Currently, there is considerable interest in measuring the conformational stability of globular proteins. Most often, estimates of the conformational stability are based on an analysis of denaturant-induced or thermal unfolding curves or by calorimetric studies. There are two methods commonly used to analyze the protein unfolding transition curves, linear extrapolation and nonlinear least-squares fitting. The former was used in this study.

Assuming a two-state model, the denaturation appears as a sigmoidal curve consisting of three regions: pretransition, transition and posttransition regions. A typical example is given in Figure 9A. The pretransition and posttransition regions

Figure 9: The linear extrapolation method for an example of thermal denaturation curve. A) Extrapolation of the linear portions of pretransition and posttransition regions to determine the values for $y - y_N$ and $y_D - y$ from raw data.; B) resulting fraction of unfolded molecules (F_u) from A where $F_u = (y - y_N) / (y_D - y_N)$.

may show a temperature dependence, but it is assumed that any temperature dependent changes in these regions are simply due to the temperature dependence of chromophore signal in the native and denatured protein respectively and not due to any structural change on the protein itself. The transition region denotes those conditions where both the native and unfolded forms of the protein are in equilibrium. In order to evaluate the data, a two-state model is assumed and all data are converted into the fraction of unfolded molecules (F_u) by the following relationships (see Figure 9B):

$$[N] = y_D - y \quad (9)$$

and

$$[U] = y - y_N \quad (10)$$

By combining equation 9, 10

$$[N] + [U] = y_D - y_N \quad (11)$$

Therefore,

$$F_u = y_D - y / y_D - y_N \quad (12)$$

where y is the signal amplitude observed within the unfolding transition; y_D and y_N are the characteristic signal amplitudes of the native and denatured states of the protein, respectively, where y is measured. The values of y_D and y_N within the transition region are obtained through the linear extrapolation analysis of the posttransition and pretransition regions, respectively.

For a two-state transition, the equilibrium constant is defined as follows:

$$K_{eq} = [U] / [N] \quad (13)$$

In terms of the fraction of unfolded molecules equation 13 becomes:

$$K_{eq} = F_u / 1-F_u \quad (14)$$

The free energy of unfolding, ΔG , is calculated for each point in the transition using the following relationship:

Since

$$\Delta G = -RT \ln K_{eq} \quad (15)$$

then

$$\Delta G = -RT \ln(F_u / 1-F_u) \quad (16)$$

For analyzing thermal unfolding curves, a least-squares analysis of plot of ΔG as a function of temperature was used to determine the transition midpoint (T_m), the temperature where $\Delta G = 0$, that is, where $F_u = 0.5$.

From data analysis, one thermodynamic parameter the transition midpoint, can be obtained which provides a measure of the stability of the protein. The higher transition midpoint, the more stable the protein is. The T_m is the temperature required to yield the equal numbers of folded and unfolded molecules. All fluorescence and CD data obtained in this study were evaluated by this

method. Microsoft Excel™ was used to perform all linear extrapolation analyses and T_m calculations.

Chapter 3: RESULTS AND DISCUSSION

Thermal denaturation of proteins can be followed as a function of temperature by the use any of observable parameter which shows a detectable difference between the native and unfolded states of the protein. The unfolding of RNase A was monitored by fluorescence and circular dichroism at pH* 3.0, 0.1 M KCl. Each technique monitors a different property of the protein during the unfolding process. By comparing these data with DSC and ANS data from previous studies, we have further characterized the intermediate structures present in the unfolding process and have gleaned information about their structures and relative stabilities.

3.1 Thermal Denaturations Monitored by Fluorescence

Fluorescence spectra of RNase A in 50% aqueous-methanol were collected at intervals of 60 seconds at temperatures between -10 and 70 °C. A small linear increase in fluorescence is observed before and after the transition under all conditions. As a general example of fluorescence raw data, Figure 10 shows the thermal transition curve of 20 μ M RNase A at pH* 3.0 in aqueous buffer. At low temperature, there is a characteristic temperature effect on the molar fluorescence of tyrosine residues. At higher temperatures, 40-50 °C, the fluorescence increase

Figure 10: The thermal denaturation of 20 μ M RNase A at pH* 3.0 (0.1 M KCl), monitored by fluorescence (280/305 nm). Data is given as the fluorescence intensity vs. temperature.

corresponds to the denaturation of the protein. The posttransition region (50-75 °C) shows the characteristic temperature effect on the molar fluorescence of tyrosine residues. The posttransition region is noticeably steeper than the pretransition region. This is due to the fact that the denatured protein has a higher fluorescence than the native state and thus the temperature effect on such fluorescence is correspondingly greater in magnitude.

Subsequent plotting of the fraction of unfolded molecules versus temperature gave sigmoidal curves from which the T_m values were calculated. Figure 11, 12, 13, 14 give the results in 0 M, 0.5 M, 1.0 M, 1.5 M urea, respectively. Each condition was examined at least three times. The average value of the midpoint of thermal transition in aqueous buffer is 44.5 °C (Figure 10). The average values for the midpoints of thermal transitions in 50% methanol in various concentrations of urea are given in Table 2. All thermal unfolding experiments performed here were fully reversible (data not shown). The results of fluorescence experiments show that T_m value decreases as the concentration of urea increases.

3.2 Thermal Denaturations Monitored by CD

The unfolding transition for RNase A was also examined via far UV-CD by measuring the ellipticity at 220 nm which monitors changes in α -helix content. Figure 15 gives the CD data of thermal denaturation of RNase A in aqueous buffer (0.1 M KCl, pH* 3.0). The sharp transition is consistent with a two-state process.

Figure 11: The thermal denaturation of 20 μ M RNase A in 50% aqueous-methanol at pH* 3.0 (0.1 M KCl), monitored by fluorescence (280/305 nm). Data is given as the fraction of unfolded molecules (Fu) vs. temperature.

Figure 12: The thermal denaturation of 20 μ M RNase A in 50% aqueous-methanol in the presence of 0.5 M urea at pH* 3.0 (0.1 M KCl), monitored by fluorescence (280/305 nm). Data is given as the fraction of unfolded molecules (Fu) vs. temperature.

Figure 13: The thermal denaturation of 20 μ M RNase A in 50% aqueous-methanol in the presence of 1.0 M urea at pH* 3.0 (0.1 M KCl), monitored by fluorescence (280/305 nm). Data is given as the fraction of unfolded molecules (Fu) vs. temperature.

Figure 14: The thermal denaturation of 20 μ M RNase A in 50% aqueous-methanol in the presence of 1.5 M urea at pH* 3.0 (0.1 M KCl), monitored by fluorescence (280/305 nm). Data is given as the fraction of unfolded molecules (Fu) vs. temperature.

Table 2: Transition Midpoints (T_m) for the Thermal Denaturation of RNase A

	Aq. buffer	50% MeOH	0.5M urea ²	1.0M urea ²	1.5M urea ²
Fluorescence	44.5 ± 0.5	27.4 ± 0.4	26.6 ± 0.4	23.6 ± 0.9	22.4 ± 0.8
CD	43.9 ± 0.4	37.4 ± 0.3	33.3 ± 0.7	30.1 ± 0.3	22.4 ± 0.3
Calorimetry ¹	44.0	27.0	25.6	22.2	
			34.2	32.6	
ΔH (kcal/mole) ¹	78.5	67.0	56.0	60.0	
			37.0	30.0	

¹ Taken from E. Tiktopulo, unpublished data.

² Solutions were in 50% methanol (pH* 3.0, 0.1 M KCl) in the presence of urea.

³ Aq. buffer and MeOH stand for aqueous buffer and methanol respectively.

Figure 15: The thermal denaturation of 20 μM RNase A in aqueous solution at pH* 3.0 (0.1 M KCl), determined by circular dichroism at 220 nm. Data is given as $\Delta\epsilon$ vs. temperature.

The reduction in the absolute value of the CD signal reflects the loss of α -helix with increasing temperature. At temperatures higher than 45 °C, the signal is close to -0.3 AU/M cm. This is pretty close to zero which is the signal obtained for the protein when completely denatured by 6M GdmCl.

Each experiment was done at least three times. The average value of the midpoint of thermal transition in aqueous buffer is 43.9 °C (Figure 15). Figure 16, 17, 18, 19 give the CD-monitored thermal transitions of RNase A at pH* 3.0, 0.1 M KCl in 50% methanol in the presence of 0 M, 0.5 M, 1.0 M, 1.5 M urea respectively. The average values of the transition midpoints are given in Table 2. The urea-induced folding transition is sharp indicating a cooperative process which can be defined as a two-state transition. All thermal unfolding under these conditions were fully reversible (data not shown).

Figure 20 gives the CD-monitored thermal denaturation of RNase A in 50% methanol in the presence of 1.5 M urea and is representative of all other transitions in the presence of urea. From Figure 20, one can see that for the unfolded state, the intensity of the CD signal is -3.0 AU/M cm at the end of transition. Also, signals at the end of transition associated with other conditions are -2.7, -3.3, -3.0 AU/M cm in 50% methanol with 0.0 M, 0.5 M and 1.0 M urea, respectively. In contrast, RNase A denatured by 6 M GdmCl and the aqueous thermal denaturation give a CD signal close to zero indicating that significant residual secondary structure is present in 50% methanol solutions. This indicates

Figure 16: The thermal denaturation of 20 μ M RNase A in 50% aqueous-methanol at pH* 3.0 (0.1 M KCl), monitored by CD at 220 nm. Data is given as the fraction of unfolded molecules (Fu) vs. temperature.

Figure 17: The thermal denaturation of 20 μ M RNase A in 50% aqueous-methanol in the presence of 0.5 M urea at pH* 3.0 (0.1 M KCl), monitored by CD at 220 nm. Data is given as the fraction of unfolded molecules (F_u) vs. temperature.

Figure 18: The thermal denaturation of 20 μ M RNase A in 50% aqueous-methanol in the presence of 1.0 M urea at pH* 3.0 (0.1 M KCl), monitored by CD at 220 nm. Data is given as the fraction of unfolded molecules (Fu) vs. temperature.

Figure 19: The thermal denaturation of 20 μ M RNase A in 50% aqueous-methanol in the presence of 1.5 M urea at pH* 3.0 (0.1 M KCl), monitored by CD at 220 nm. Data is given as the fraction of unfolded molecules (Fu) vs. temperature.

Figure 20: The thermal denaturation of 20 μM RNase A in 50% aqueous-methanol in the presence of 1.5 M urea at pH* 3.0 (0.1 M KCl), determined by circular dichroism at 220 nm. Data is given as $\Delta\epsilon$ vs. temperature.

that some secondary structure is retained in the unfolded structure, in particular α -helix. Studies by Lustig and Fink (1992) show that in the presence of 50% methanol, thermally denatured RNase A retains about 14% α -helix (and a comparable amount of β -sheet). Further support for some residual structure in the thermally denatured RNase A in 50% methanol comes from proton NMR studies at pH* 3.0, 50% methanol (Biringer and Fink, 1982).

3.3 Noncoincident Thermal Denaturation Transitions

In order to compare the effect of urea on fluorescence and CD transitions, the T_m values for both measurements were plotted versus the concentration of urea (Figure 21). This plot shows that the T_m values obtained from CD are higher than those of corresponding fluorescence measurements, except at 1.5 M urea where they are the same. Thus, with the exception of the 1.5 M urea data, the loss of the secondary structure occurs at higher temperatures than the loss of the tertiary structure at low concentration of urea. This is consistent with a stepwise unfolding of the protein where the tertiary structure is lost at lower temperatures than the secondary structure in the presence of methanol. The coincidence of the transition at 1.5 M urea indicates that high concentrations of urea destabilizes the secondary structure to the point that it is no longer stable more than the tertiary structure. The plot shows a relatively linear relationship between T_m and the concentration of urea from both data sets. The slope of CD data is -9.7 and that of

Figure 21: Noncoincidence of unfolding transition in the thermal denaturation of RNase A in 50% aqueous-methanol at pH* 3.0 in the absence or presence of urea. Circles represent the data obtained from fluorescence measurements. Triangles represent the data obtained from CD measurements.

fluorescence is -3.6. The steeper CD curve indicates that urea destabilizes the secondary structure to a greater degree than the tertiary structure. This suggests that urea destabilizes the structure by competing for hydrogen bonds rather than through hydrophobic interactions as the tertiary structure is destabilized primarily by hydrophobic interactions and the secondary structure by hydrogen bonding.

3.4 Comparison of the data obtained from different spectroscopic techniques

A summary of the data obtained from this project is compared with corresponding data obtained from different techniques in Table 2. With the exception of the CD data obtained in 50% methanol in the absence of urea, the T_m values obtained from fluorescence and CD show coincidence with Tiktopulo's calorimetric data. Calorimetric data obtained in the presence of urea show two unfolding transitions. Each of these has a spectroscopic counterpart. The fluorescence data compares well with the lower temperature transition and the CD data compares well with the high temperature transition. These results indicate that the lower temperature transition corresponds to the loss of the tertiary structure and the high temperature transition corresponds to the loss of the secondary structure. Such results indicate that a molten globule state is populated between these transition temperatures.

In 50% methanol and the absence of urea, the calorimetric data shows only a single unfolding transition. However, spectroscopic data presented here and elsewhere indicate two transitions are observable under these conditions. The data obtained for the thermal denaturation of RNase A in 50% methanol in the absence of urea fall into two classes, those with a midpoint near 27 °C and those with a midpoint near 38 °C. Both the UV absorbance data at 286 nm (Biringer and Fink, 1982) and our fluorescence data have essentially the same T_m in the vicinity of 27 °C. Proton NMR data (Biringer and Fink, 1982) shows two transitions under these conditions. The relative peak areas for C-2 protons of the four histidine residues in the native state in 50% methanol all show a major transition in the vicinity of 37 °C; His-48 and -105 also show transitions in the vicinity of 27 °C, whereas His-12 and -119 show pre-transitions at lower temperatures. Lustig and Fink's data (1992) also show that a two-state transition is observed with a midpoint at 38 °C for α -helix and an additional at 27 °C from dynamic light scattering experiments. Taken together, the thermal unfolding of RNase A in 50% methanol at pH* 3.0, 0.1 M KCl occurs in two major transitions, one center around 27 °C and the other in the vicinity of 38 °C. Therefore, the overall unfolding occurs over a very board range of temperature. The lower temperature transition is detected by probes sensitive to the tertiary structure of the protein, such as absorbance and fluorescence which monitor the tyrosine sidechain environment. The higher temperature transition is

associated with the loss of α -helix as observed by CD and the NMR signal corresponding to His-12 which is located within the N-terminal helix.

The fact that the calorimetric data shows only one a single transition in the absence of urea and dual transitions in the presence of urea indicates that urea interacts with a portion of the protein's native structure. Since the additional transition is associated with the loss of α -helix, it can be concluded that interaction involves regions of the native α -helix on the protein.

In the presence of both 50% methanol and 1.5 M urea, the fluorescence and CD data are coincident indicating that the secondary structure is lost at the same temperature as the tertiary structure and the molten globule states are not populated. Unfortunately, there is no calorimetric data for the corresponding set of conditions. However, Tiktopulo did perform calorimetric experiments under very similar conditions, in particular, in the absence of 0.1 M KCl. Comparing the data obtained in the presence of 0.1 M KCl given in Table 1 it can be readily seen that the higher ionic strength has only a small effect on the transition temperature and no effect on the number of observed transitions. For this reason, it seems logical to assume that a single calorimetric transition should be expected for the denaturation in the presence of both 50% methanol and 1.5 M urea at 1.0 M KCl. Armed with this assumption and the data for lower concentrations of urea, it seems logical to conclude that urea serves to destabilize both the tertiary structure

and α -helix whereas methanol destabilizes the tertiary structure and stabilizes α -helix. This strongly suggests that methanol and urea interact with α -helix in different ways which is contrary to the belief held by many in the protein folding field that urea denatures proteins through primarily hydrophobic interactions.

A simple three species model (Figure 22) is proposed to describe these results. In this model, the presence of methanol favors the native (N) to the molten globule (M) state transition whereas urea favors the native to the unfolded (U) state transition.

Figure 22: Three species mechanism. N represents the native state, U represents the unfolded state and M represents the molten globule state.

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