1997

Denaturation studies of RNase A using monohydric alcohol

Kameswari Aysola
San Jose State University

Follow this and additional works at: https://scholarworks.sjsu.edu/etd_theses

Recommended Citation

Aysola, Kameswari, "Denaturation studies of RNase A using monohydric alcohol" (1997). *Master's Theses*. 1420.

DOI: <https://doi.org/10.31979/etd.qy46-t26d>
https://scholarworks.sjsu.edu/etd_theses/1420

This Thesis is brought to you for free and open access by the Master's Theses and Graduate Research at SJSU ScholarWorks. It has been accepted for inclusion in Master's Theses by an authorized administrator of SJSU ScholarWorks. For more information, please contact scholarworks@sjsu.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

DENATURATION STUDIES OF RNase A USING
MONOHYDRIC ALCOHOL

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
Kameswari Aysola
May, 1997

UMI Number: 1384669

UMI Microform 1384669
Copyright 1997, by UMI Company. All rights reserved.

**This microform edition is protected against unauthorized
copying under Title 17, United States Code.**

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

Dr. Roger G. Bringer

Dr. Leon Yengoyan

Dr. Karen Singmaster

APPROVED FOR THE UNIVERSITY

ABSTRACT

The native conformation of a protein is maintained through a delicate balance between various noncovalent forces. It has been shown that both hydrogen bonding and the hydrophobic effect are important for the stability of a protein. We have examined the concentration-mediated denaturation of bovine pancreatic ribonuclease (RNase A) using monohydric alcohols at constant temperature and at constant protonic activity (pH^*) of both 3.00 and 4.00.

The results of ethanol-mediated denaturation at pH^* 3.00 showed the expected increase in fluorescence emission indicating a loss in tertiary structure. The change in the CD signal at 222 nm gave an unexpected increase indicating an increase in the secondary structure, in particular, α -helix.

The results of t-butyl alcohol-mediated denaturation at pH^* 3.00 showed the expected increase in fluorescence emission at lower alcohol concentration. At higher alcohol concentrations, the data was scattered due to the formation of soluble aggregates. The change in the CD signal at 222 nm gave an unexpected increase with increasing alcohol concentration indicating an increase in the secondary structure, in particular α -helix. Such changes are consistent with the formation of a molten globule state.

Denaturation studies of RNase A with both the alcohols at pH^* 4.00 show no significant change in either tertiary structure or secondary structure.

ACKNOWLEDGMENTS

I wish to gratefully acknowledge Dr. Biringer, whose advice and guidance has been invaluable during the course of this thesis.

I wish to thank Dr. Yengoyan and Dr. Singmaster for their contributions.

Finally, I would like to express my gratitude to my husband, Kumar, who patiently suffered through all this and our daughter Monica, who was born amidst all this.

TABLE OF CONTENTS

	Page
ABSTRACT.....	iv
ACKNOWLEDGMENT.....	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
LIST OF ABBREVIATIONS.....	x
 1. INTRODUCTION.....	 1
1.1. SIGNIFICANCE OF PROTEIN FOLDING.....	1
1.2. STRUCTURAL STATES OF PROTEINS.....	2
1.3. FORCES THAT STABILIZE PROTEIN STRUCTURE.....	5
1.4. MODELS FOR PROTEIN FOLDING.....	12
1.5. METHODS TO EXAMINE PROTEIN FOLDING.....	19
1.6. MODEL PROTEIN RNASE A.....	21
 2. MATERIALS AND METHODS.....	 23
2.1. MATERIALS.....	23
2.2. PURIFICATION OF RNASE A.....	23
2.3. PREPARATION OF PROTEIN STOCK SOLUTION.....	23
2.4. PREPARATION OF STOCK INDICATOR SOLUTIONS.....	24
2.5. AQUEOUS STANDARD CURVE.....	25
2.6. DETERMINATION OF PAH* FOR AQUEOUS/ORGANIC SOLUTIONS	26
2.7. PREPARATION OF DENATURANT DILUTION SERIES.....	30

2.8. FLUORESCENCE-MONITORED CHEMICALLY-INDUCED DENATURATION TRANSITIONS.....	31
2.9. CD-MONITORED CHEMICALLY-INDUCED DENATURATION TRANSITIONS.....	32
2.10. DATA ANALYSIS.....	33
3.0. RESULTS AND DISCUSSION.....	35
3.1. PROTONIC ACTIVITY FOR AQUEOUS/ALCOHOL SOLUTIONS.....	35
3.2. DENATURATION OF RNASE A BY MONOHYDRIC ALCOHOLS.....	38
3.3 TRANSITION MIDPOINTS FOR THE OVERALL SERIES OF ALCOHOLS.....	47
3.4 AGGREGATION.....	50
4.0 CONCLUSIONS.....	53
REFERENCES.....	54

LIST OF TABLES

Table	Page
1. Transition Midpoints for the Fluorescence-monitored denaturation of RNase A at $\text{pH}^* 3.00$	49

LIST OF FIGURES

Figures	page
1. Hydrogen bonding groups.....	10
2. Diffusion-collision model.....	15
3. Hydrophobic zipper model.....	16
4. Sequential mechanism.....	18
5. Aqueous standard curve at pH 3.00.....	27
6. Aqueous standard curve at pH 4.00.....	28
7. Final pH vs M for ethanol.....	36
8. Final pH vs M for t-butyl alcohol.....	37
9. Fluorescence and CD for ethanol at paH* 3.00.....	40
10. Fluorescence of N-acetyl tyrosine ethyl amide in ethanol.....	42
11. Fluorescence and CD for ethanol at paH* 4.00.....	43
12. Fluorescence and CD for t-butyl alcohol at paH* 3.00.....	45
13. Fluorescence and CD for t-butyl alcohol at paH* 4.00.....	48
14. Transition midpoints at paH* 3.00.....	51

LIST OF ABBREVIATIONS

Asn	Asparagine
CI	Compact intermediate
CD	Circular dichroism
C _m	Concentration midpoint
Far UV-CD	Far ultraviolet circular dichroism
Glu	Glutamic acid
His	Histidine
HZ Model	Hydrophobic Zipper model
N	Native state
NMR	Nuclear magnetic resonance
paH*	-log Protonic activity
RNase A	Bovine pancreatic Ribonuclease A
RNase T1	Ribonulcease T1
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Trp	Tryptophan
U	Unfolded state
UV	Ultraviolet

1. INTRODUCTION

Proteins are complex biological macromolecules and constitute the largest fraction of a cell's content, besides water. Proteins are the building blocks of life and play important roles in all biological processes. There are many kinds of proteins found in cells. Each has its own characteristic amino acid sequence, three-dimensional structure, and a specific function. Some proteins have catalytic activity and function as enzymes; some serve as structural elements; some carry specific signals as in the case of receptors and some carry specific substances in and out of the cells. Proteins are known to be biologically active only when they are in their native structure. The mechanism by which a protein attains its biologically active three-dimensional native structure is the question at hand and is not a very well understood problem.

1.1 SIGNIFICANCE OF PROTEIN FOLDING

The fact that proteins with a given amino acid sequence are found to attain the same native structure, and different amino acid sequences lead to different three-dimensional native structures indicates that the amino acid sequence of a polypeptide chain "codes" for its native structure. However, it is not understood how the amino acid sequence "codes" for its native structure. Deciphering this "code" is known as the protein folding problem. Once this "code" is deciphered, one could directly translate the amino acid sequence into a three-dimensional native structure. This is particularly important for the Human Genome project, as the Human Genome project will yield primary structures for many proteins with

unknown structure and function. Knowledge of the protein folding "code" is of paramount importance to the interpretation of the results, as conventional methods for structure determination by X-ray crystallography and NMR are far too time consuming to handle the large amount of data that will be obtained from Human Genome Project. Furthermore, an understanding of how proteins attain their native conformation will provide insight required for designing a protein for a particular function.

1.2 STRUCTURAL STATES OF PROTEINS

Proteins in all living species are constructed from the basic set of twenty amino acids. These amino acid monomers are held together in a linear array by amide linkages to form a polypeptide chain. Proteins are made up of single or multiple polypeptide chains that fold upon themselves to form a specific three-dimensional structure which in turn defines the function of the protein.

Protein structure is classified into four categories: primary structure, secondary structure, tertiary structure, and quaternary structure. The primary structure of a protein refers to the sequence or order of amino acid along the polypeptide chain. The secondary structure refers to the localized spatial arrangement of the polypeptide backbone. The three major types of secondary structures are helices, pleated sheets, and turns. Tertiary structure refers to the three-dimensional arrangement of the secondary structural elements. For proteins that consist of more than one polypeptide chain, the spatial arrangement of these polypeptide subunits is referred as quaternary structure (Creighton, 1990).

There are three conformational states of proteins that are known: the native or fully folded state (N), compact intermediate or molten globule state (CI), and the unfolded or random coil state (U). A complete knowledge of these states will provide information regarding the mechanism of protein folding, which in turn will help decipher the “code” that determines how the amino acid sequence defines the native state.

1.2.1 Native or Fully Folded State (N):

The native, fully folded three-dimensional structure of a protein is required for its biological activity. Over 500 different native protein structures are known, from x-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) studies. Proteins with the same primary amino acid sequence adopt to the same three-dimensional structure and no other alternative fully folded structures. Most of the native protein structures have the same common feature, the most nonpolar side chains are found in the interior of the protein, whereas most of the hydrophilic side-chains are located at the surface of the protein. The native conformation is compact and highly ordered. Since the tertiary structure of a globular protein is determined by its amino acid sequence, it does not require any extrinsic factors or input of energy to attain its fully folded biologically active conformation. The ordered native state is stabilized by a delicate balance between a number of noncovalent interactions. The native structure is only marginally more stable than the unfolded state and can be disrupted by changes in temperature, pH or by the addition of chemical denaturants.

1.2.1 Compact Intermediates or Molten Globule State (CI)

A number of globular proteins, such as bovine pancreatic ribonuclease A (RNase A) (Biringer et al., 1982) and α -lactalbumin (Kuwajima, 1989), can exist, under suitable conditions, in metastable intermediate conformations that are neither native nor unfolded conformations, but somewhere in between. Kuwajima proposed that intermediate states of different proteins have some common characteristics and coined the term molten globular state to refer to these intermediates. The common characteristics of these molten globular states are many. The molten globular states have a compact structure with a large content of natively like secondary structure, but have an unfolded tertiary structure. For this reason, interior side-chains of molten globular states are in a homogeneous environment, unlike in fully folded states where they are found in asymmetric environment.

Several studies have been done to characterize the role of the intermediate states (Kuwajima, 1989; Ptitsyn, 1992). Kuwajima (1989) demonstrated, in his work with α -lactalbumin, that the nonspecific interactions between the hydrophobic groups in the interior of a protein stabilize the molten globule state. Further, he also demonstrated that this molten globular state forms early in folding process and its formation is the rate limiting step.

1.2.3 Unfolded State or Random Coil (U)

The unfolded state of a polypeptide chain has no defined solution three-dimensional structure and can be best described as a random coil. In

the unfolded state, rotations about every chemical bond of the polypeptide are independent of every other bond rotations and determined only by the local stereochemistry of the polypeptide backbone.

Dill and Shortle (1991) have done extensive work on the denatured state of proteins. They suggest that it is useful to distinguish the unfolded states from the compact denatured state. The unfolded states are highly open and solvent-exposed, with little or no residual nativelike structures, and are obtained only under strong denaturation conditions. Compact denatured states are the ones with some residual native secondary structure and are obtained under weaker denaturation conditions. A number of experiments done by Tanford (1968) and by Dill and Shortle (1991) have demonstrated that under strong denaturation conditions, such as 6 M guanidinium chloride or 8 M urea, proteins unfold from their native compact structures to form unfolded structures. These unfolded structures were found to have the same hydrodynamic properties as a random coil polypeptide.

1.3 FORCES THAT STABILIZE PROTEIN STRUCTURE

The compact conformation of the native protein is stabilized by a collection of different forces. The contributing forces are as follows: electrostatic interactions, hydrogen bonding, van der Waals interactions, and hydrophobic interactions.

1.3.1 Electrostatic Interactions

Electrostatic interactions take place between charged groups, and can be either attractive or repulsive. During the early days of protein folding studies, acids and bases were among the first known protein denaturants. For this reason, stabilizing forces were assumed to be electrostatic in nature. In proteins, electrostatic interactions are dependent on the pH of the solvent and the ionic strength, where the pH determines the total charge on the protein and the ionic strength determines the extent of interaction among those charges.

The difference between the electrostatic forces of the folded and unfolded conformations of the protein will contribute towards the conformational stability of the protein. The interactions are larger in the folded state than in the unfolded state, as the charges in the folded state are closer together.

Electrostatic interactions will affect protein stability in two different ways, classical electrostatic effects and specific charge interactions. The classical electrostatic effects are the nonspecific repulsive interactions that arise when a protein is highly charged (at extremes of pH). At the isoelectric point of the protein, electrostatic interactions have little effect on the protein stability, as at their isoelectric point proteins have equal amounts of positive and negative charges (Pace et al., 1990). As the acidity or basicity of the solution is increased beyond pI, the net charge on the protein increases and the stability of the protein decreases with the corresponding increase in charge repulsion (Dill, 1990).

Protein stability is also affected by specific charge interactions. An ion pairing or salt bridging occurs when two oppositely charged amino acid side chains are in close spatial proximity. The protein stabilization due to ion pairing comes primarily from the charged groups buried in the core of the protein. Early work indicated that ion pairing is the dominant force that contributes toward protein stability (Jaenicke, 1991). In contrast, several researchers have now shown that ion pairing is not a dominant force in protein stability, since few ion pairs are buried within the core of the globular proteins. Hence, electrostatic interactions may be the major contribution towards the conformational stability of some proteins, but not in other proteins (Dill, 1990).

1.3.2 Hydrophobic Interactions

The most important noncovalent interaction that determines a protein's native conformation and stability in an aqueous solvent, is thought to be the hydrophobic effect. Hydrophobic interaction is defined in a number of ways and means different things to different people. The presently accepted definition is given in terms of the free energy of solvation. To facilitate solvation of nonpolar compounds following transfer into an aqueous solution, water molecules form a highly ordered cavity in which the nonpolar solutes will fit (Dill, 1990). The solvation is enthalpically favored, but the entropy associated with the ordering of water molecules is unfavorable to the extent that the free energy is dominated by entropy, rendering the associated free energy of the system positive and opposing solvation (Privilov and Gill, 1989). This explains

why the nonpolar residues are located in the interior of native proteins forming a hydrophobic core, whereas polar and charged amino acid residues are exposed to solvent water.

Water is a poor solvent for nonpolar compounds because of the inability of such compounds to form hydrogen bonds with water. This lack of hydrogen bonding between nonpolar molecules and water is thought to be the major factor leading to the compaction, and the structural stability of proteins (Lins and Brasseur, 1995). Upon denaturation, the hydrophobic groups become exposed to the solvent, which serves to order water molecules and decrease the solvent entropy. Compaction serves to bury such hydrophobic groups, resulting in an increase in solvent entropy and hence an overall reduction in the free energy of the solution.

There is a considerable amount of data to support hydrophobic interactions as being a major driving force in the folding of proteins. The importance of hydrophobic residues in the stabilization of proteins is also strongly supported. First, it has been shown that nonpolar solvents denature proteins, as the hydrophobic interactions reduce the free energy of the unfolded state due to the solvation of nonpolar residues. In other words, it can be said that the nonpolar solvents reduce the free energy of the unfolded state by solvating the exposed nonpolar residues and the polypeptide backbone (Dill, 1990).

1.3.3 Hydrogen Bonding and van der Waals Interactions

Hydrogen bonds arise from a hydrogen atom that is shared between two electronegative atoms. The hydrogen bond involves the interaction of a proton, carrying a partial positive charge as the donor group with the electron density on an acceptor atom. When donor hydrogen and acceptor molecules are arranged in a linear fashion, the strength of a hydrogen bond is at its maximum. But bifurcated hydrogen bonds are also possible where a hydrogen atom is directed between the two acceptors, and interacts with both.

In proteins, most of the hydrogen bonds present are those between the carbonyl oxygens and amide hydrogens of the peptide backbone. Hydrogen bonds can also be formed between hydrogen bond donors and acceptors on the side chains of some amino acids like Ser, Thr, Tyr, Asn, Glu, His, Trp (Figure 1).

In proteins, van der Waals interactions can be either attractive or repulsive. These interactions can arise either from the electrostatic interactions among fixed dipoles, such as carbonyl and amide groups of the peptide backbone, or induced dipoles where a permanent dipole, such as a carbonyl group, induces a dipole in a nearby group by electrostatically distorting its electron distribution.

Intramolecular hydrogen bonding was initially thought to be the major driving force for protein folding and stability. This idea was inspired by the original X-ray crystallographic structures, where all of the hydrogen bonding groups are arranged such that all the possible hydrogen bonds are formed. Later it was proposed that hydrogen bonds are less important

Figure 1: Hydrogen bonding groups. Hydrogen bonding groups in proteins, comprising the main chain peptide group and all the principle hydrogen bonding side chains. Reproduced with permission from E.N.Baker et al., 1984.

since they replace similar interactions with solvent molecules in the free unfolded chain, and their main role is to confer structural specificity (Jeanicke, 1991). Brandts and Hunt (1967) and others have stated that the denaturation of proteins with nonpolar solvents is due to the reduction of the free energy of the unfolded state caused by the solvation of hydrophobic residues and indicated that the hydrophobic effect is the driving force for protein folding and stability (Brandts and Hunt, 1966). On the other hand, Shirley et al., (1991) showed that both hydrogen bonding and hydrophobic effect make comparable contributions to the conformational stability of ribonuclease T1. This is further shown in denaturation studies of RNase A done by Biringer's group (unpublished data) using alkyl ureas, where it is clear that both the hydrophobic effect and hydrogen bonding are important for the stability of proteins.

Concluding Remarks:

A protein's native conformation is based on a delicate balance between different forces. Hydrogen bonding, van der Waals forces, and electrostatic forces as well as hydrophobic forces are important for the stability of the proteins. The relative importance of hydrogen bonding and the hydrophobic effect in the stabilization of proteins is unknown. But, several studies show that hydrophobic interactions between the nonpolar groups are the dominant forces in the stabilization of the native compact protein structure (Dill, 1990; Lins and Brasseur, 1995; Privilov and Gill, 1989).

1.4 MODELS FOR PROTEIN FOLDING:

As discussed above, the amino acid sequence of a protein contains the code for the unique three-dimensional native conformation, which is biologically active. The "code" can be understood if the folding pathways for a representative number of proteins are well defined. Folding pathways can be understood by examining and characterizing the structures of partially folded states along each pathway.

A number of models have been proposed to describe the protein folding pathways. These models can be classified into two groups, namely, structural models and kinetic models. In the structural models, the characterization of the native, partially folded, and unfolded structures are emphasized and thought to be important in order to understand the protein folding process completely. But, structural models are not specific about the factors that control the rate of folding. The structural models are difficult to come up with, as gathering structural information is intrinsically slow, and requires that intermediates be highly populated; this is difficult to do experimentally. Kinetic models emphasize the factors that control the rate of folding without being too specific about the structures of the intermediates. Several kinetic models have been proposed to explain the folding process (Kim and Baldwin, 1982).

1.4.1 Biased Random Search Model

One of the first models proposed was the Biased Random Search Model, where the folding process was hypothesized to consist of a purely random search of all possible conformations. But this hypothesis was

dismissed, as the time required for folding a very small protein with 100 amino acid residues would be around 10^{50} years which is larger than the age of the known universe (Karplus and Weaver, 1976). However, Levitt (1976) showed, by means of a computer-simulated folding for a lattice model, that the number of possible chain conformations is drastically reduced if only self avoiding conformations are allowed. Based on this result and others, Levitt proposed that a biased random search may play an important role during the early stages of folding.

1.4.2 Nucleation Growth Model

The Nucleation-Growth Model proposes that proteins fold by means of a nucleation center that is formed in the early stages of the folding pathway, through random conformational fluctuations of the unfolded protein. This center acts as a template on which protein folding proceeds. This nucleation center is not observable as a populated species, either because folding occurs rapidly after the nucleation or because the nucleus is unstable by itself and breaks down if it is not stabilized by further folding (Wetlaufer, 1973). This model does not account for the existence of intermediates observed in the folding of number of proteins nor does it explain as to how the nucleation center achieves its conformation. For these reasons the Nucleation-Growth Model is not accepted as a complete model.

1.4.3 Diffusion-Collision-Adhesion Model

The Diffusion-Collision Model was introduced in 1976 by Karplus and Weaver. According to this model, in the early stages of folding short segments of the unfolded protein chain fold independently into microdomains which diffuse together and adhere, resulting into a large structure (Figure 2). The process of protein folding involves a series of sequential diffusion-collision steps. The microdomains which form in the early stage of protein folding are unstable, but become stable upon completion of folding. Karplus and Weaver (1994) calculated the rate to fold by means of a diffusion collision model for a protein (apoMB) and found that the diffusion and collision to form the microdomains would be the rate limiting step for this protein. However, Kim and Baldwin (1982) studied kinetics of folding of RNase A and proposed that adhesion or coalescence of the two microdomains may be the rate-limiting step in this model.

1.4.4 Hydrophobic Zipper Model

Hydrophobic Zipper (HZ) Model was proposed by Dill et al., (1993). According to the HZ Model, folding begins with the hydrophobic monomers (side chains) pairing together and then these hydrophobic contacts act as constraints that bring other contacts into spatial proximity. These contacts further constrain the chain and "zip" up the next contacts. For any amino acid sequence, chain folding may nucleate at different sites and follow different zipper paths in parallel, which converge to form a hydrophobic core (Figure 3). Hydrophobic zipper collapses because the folding of a heteropolymer is a highly cooperative process. This

Figure 2: Diffusion-Collision Model. A Cartoon representation of diffusion-collision folding kinetics of a small globular proteins or protein domains. The microdomains (A-G) (indicated by dashed outlines) are individually unstable. The stable coalescent intermediates (indicated by solid outlines) are held together by hydrophobic interactions. Reproduced with permission from Karplus and Weaver, 1994.

Figure 3: Hydrophobic-Zipper (HZ) model. A cartoon representation of HZ model for protein folding pathways. The closest hydrophobic (H) residues (solid dots) in sequence pair together first, constrain the chain and bring other H monomers into spatial proximity. As H contacts form and develop a core, helices and sheets zip up if they have appropriate H sequences. Reproduced with permission from Dill et al., 1993.

cooperative behavior is due to the non local interactions mediated by the solvent. Further, this chain collapse may result in the formation of secondary structures such as helices, sheets etc. Dill et al., (1993) tested the HZ Model on bovine pancreatic trypsin inhibitor and cramin through computer modeling. The best structures predicted from this work have energies close to native structures. HZ Model is promising, but further research is required to confirm the validity of this model.

1.4.5 Sequential Folding Model

According to the sequential folding model, protein folding proceeds through a sequence of unique and well defined intermediate structures which may be well populated under suitable folding (Kim and Baldwin, 1982). Currently there are two popular working models possible, the framework model and the modular assembly model. According to the framework model, the secondary structures are formed before the final tertiary structure is formed (Kim and Baldwin, 1982). Ptitsyn described framework model as a hierarchical process where the fluctuating secondary structures are formed in an unfolded chain, which will merge with the preexisting blocks to form the intermediates, and finally these intermediates will readjust to form the final native tertiary conformation (Figure 4) (Ptitsyn, 1987). According to modular assembly model, protein folding begins with the independent folding of separate regions of protein molecule at different times (Baldwin, 1975; Ptitsyn, 1987). The folding process of a protein may combine features of both the framework model and modular assembly model, where formation of secondary structures

Figure 4: Sequential Mechanism. A cartoon representation of sequential mechanism of protein folding. U, completely unfolded state (random coil); S, coil with fluctuating secondary structure; I, Intermediate globular state; N, native state. Reproduced with permission from O.B.Ptitsyn, 1987.

may precede tertiary interactions, while separate domains may fold at different times as in modular assembly model (Kim and Baldwin, 1982).

1.5 METHODS TO EXAMINE PROTEIN FOLDING

Numerous methodologies have been used to examine the denaturation and renaturation processes of proteins. Chief among them are absorbance spectroscopy and fluorescence spectroscopy, which serve to measure gross changes in the tertiary structure of a protein, and circular dichroism which serves to measure the secondary structure content of a protein. Although these methods have proven invaluable in the study of protein folding, the information that they yield does not provide a complete picture of the process.

1.5.1 Absorbance and Fluorescence Spectroscopy

The spectroscopic methods are the most common methods used to study the protein folding process. The absorbance and fluorescence of the aromatic side chains reflect the environment around them. Hence, by observing the spectral changes, a wealth of information can be obtained the changes in the tertiary structure of proteins. The spectral properties change because the absorbance values for the aromatic residues are different when they are buried in the hydrophobic core of the native folded state of the protein as opposed to when they are exposed to the polar solvent in the unfolded state of the protein. In thermal denaturation studies, absorbance spectroscopy is useful and gives valuable information. But in chemical denaturation studies, absorption spectroscopy is

problematic, as a number of denaturants are found to have significant absorption in the near UV region.

Fluorescence spectroscopy can also serve as a valuable tool, as it provides information about the tertiary structural changes and dynamics of proteins. The aromatic amino acids tryptophan and tyrosine act as intrinsic fluorescent probes in proteins. In the native compact conformation of the protein, the fluorescence of these probes is lower than when the protein is in its unfolded state where these fluorescent probes are exposed to the solvent. This is due to the fact that in the compact native structure, the side chains are more efficiently quenched in the hydrophobic core of the protein than in the unfolded state of the protein where aromatic groups are exposed to the solvent. The excited state lifetime associated with fluorescence is considerably longer than that associated with absorbance, rendering fluorescence spectroscopy more sensitive to changes in environment than absorbance spectroscopy (Stryer, 1968).

The fluorescence spectra of RNase A can give a wealth of information about the changes in its tertiary structure, as it provides a measure of the solvent exposure of the tyrosine residues. RNase A does not have tryptophan. The tyrosines are exposed to the solvent upon denaturation resulting in an enhancement of the fluorescence emission compared to the fluorescence emission of the native state. The major difference in the fluorescence spectra occurs at 305 nm (emission wavelength) with an excitation wavelength of 280 nm.

1.5.2 Circular Dichroism

Circular Dichroism (CD) is a measure of the unequal absorption of left handed and right handed circularly polarized light. This is a property of all chiral molecules. In the near UV region (260-320 nm), the spectra gives information about the tertiary structure of the proteins. As mentioned above, aromatic amino acids absorb strongly in the near UV region. Although the free amino acid side chains are not chiral, when the side chains are buried in the protein structure, the π - electron cloud can be distorted in a chiral manner to give large CD signal. Upon denaturation of a protein, the π - electron cloud distortion is removed and the CD signal is lost.

The far UV CD spectra contains a wealth of information about the secondary structures of the proteins. For example, the molar ellipticity at 222 nm measures mainly the α -helix content in the protein, as it is the major contributor at this wavelength. The ellipticity at 201 nm is due to all other secondary structures, as α -helix does not contribute at this wavelength. Hence the ellipticity at 201 nm provides information about the content of secondary structures other than α -helix.

1.6 MODEL PROTEIN RNase A

Bovine pancreatic ribonuclease A (RNase A) was used as a model protein to accomplish the goals of the project. RNase A is a well studied and well characterized small globular protein. The biological function of RNase A is to catalyze the hydrolysis of ribonucleic acid. RNase A consists of a single polypeptide chain of 124 amino acid residues with four

disulfide linkages, four prolines, and six tyrosines. The x-ray crystallographic structure shows that three tyrosine residues (Tyr- 115, -76, and -73) are solvent exposed and the other three tyrosines are located in the hydrophobic core of the protein. RNase A undergoes reversible unfolding without loss of catalytic activity. RNase A is a very hydrophilic protein and tends not to aggregate in moderate concentration even in the unfolded state. Hence RNase A serves as an excellent model compound to investigate the protein folding process.

2. MATERIALS AND METHODS

2.1 MATERIALS

Bovine pancreatic Ribonuclease A was purchased from Sigma Chemical Company, St. Louis. Ultra pure guanidinium chloride was purchased from Schwartz-Mann Biotech. Bromocresol green and 2,6-dinitrophenol were purchased from Aldrich. The HPLC grade alcohols, ethanol, 2-methyl-2-propanol, 2-methyl-1-propanol, and 2-butanol were purchased from Fisher.

2.2 PURIFICATION OF RNase A

Bovine pancreatic ribonuclease A was purified to remove any aggregated protein and RNase S by using a Sephadex™ SPC-25 ion-exchange with 0.15M phosphate buffer at pH 6.5 as the eluent (Biringer and Fink, 1982). In a typical purification process, 150 mg of RNase A was dissolved in 2-3 mL of the eluting buffer, loaded onto a pre-equilibrated column, and eluted at room temperature. The pure monomeric fractions of RNase A were pooled and dialyzed twice against 20 L of distilled deionized water. The first dialysis was performed for 2 hours and the second for another 24 hours at 4 °C. The dialyzed product was lyophilized and then stored at -20 °C.

2.3 PREPARATION OF PROTEIN STOCK SOLUTIONS

Stock protein solutions were prepared by dissolving purified RNase A in a 0.05 M acetate buffer at pH* 4.00 or in a 0.05 M formate buffer at

pH* 3.00 to a concentration of 5 mg / mL. The protein solution was mixed slowly by magnetic stirring. The protein stock solution was then filtered using 0.45 μm Millipore™ teflon filters to remove dust. The protein concentration was checked spectrophotometrically and adjusted if necessary. A 50 μL aliquot of stock protein solution was added to 800 μL of aqueous 0.1 M acetate buffer at pH 6.00 and stirred well, and the absorbance measured at 278 nm. The protein stock solution concentration was calculated using the values, MW: 13,800 , ϵ_{278} : 9800 AU/M.cm (Sage and Singer, 1962).

2.4 PREPARATION OF STOCK INDICATOR SOLUTIONS

2.4.1 Introduction

The effective proton concentration (protonic activity, where $-\log[\text{protonic activity}] = \text{pH}^*$) of mixed aqueous solutions with protic solvents (e.g. alcohols) will differ from that measured by the pH meter. This is due to the fact that protic solvents contribute significantly to the hydrogen ion equilibrium. This contribution to the hydrogen ion equilibrium is further dependent on the concentration of the cosolvent. Since protein stability is pH dependent, it is important to make sure that all the solutions used in the experiment have the same pH^* . The pH electrode contains only aqueous reference solution, which is good for measurements of the effective proton concentration in aqueous solutions, but is not suitable for solutions with cosolvents. For this reason, the protonic activity of each aqueous / organic solutions was determined using the indicator method outlined by Douzou (1977).

In order to use the indicator method to determine the actual protonic activity of a solution, one needs to choose an indicator which has distinct spectral bands for both the protonated and deprotonated forms. For a particular pH, the best choice of indicator is the one which has pK close to the desired pH. For our experiments, we chose bromocresol green and 2,6-dinitrophenol for pH^* 4 and 3 respectively.

For each pH, using the chosen indicators, an aqueous standard curve was established first, which provided a spectroscopic measure of the protonic activity. A series of aqueous buffer / indicator solutions were prepared as a function of pH and the spectra for all the solutions were measured. For aqueous solutions, the protonic activity is the same as the pH^* (pH measured by the pH meter). The pH^* of the aqueous / organic solutions were determined by the comparison of the absorbance spectra for indicator in the particular aqueous / alcohol solutions, to those from the aqueous standard curve.

2.5 AQUEOUS STANDARD CURVE

An aqueous standard curve for pH^* 4 was established using bromocresol green as the indicator. First, 50 mL of 0.05 M acetic acid solution was prepared in a volumetric flask. A 500 μL of the acetic acid solution was removed from the volumetric flask and replaced with 500 μL of the indicator stock solution (1 mg / mL). The solution was well mixed by inverting the flask several times. The diluted indicator stock solution was titrated to pH 2.0 using small amounts of 1 M HCl. A 3.00 mL aliquot of solution was removed and placed into a clean, labeled test tube.

The remaining solution was carefully titrated to pH 3.2 using 1 M NaOH, and 3.00 mL aliquot was removed and placed into a clean, labeled test tube. Using the same procedure, a series of solutions were prepared at pH values of 2.0, 3.2, 3.4, 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 6.0. At pH 2.0, 100% of the protonated form of bromocresol green is present and at pH 6.0, 100% of ionized form of bromocresol green is present. For each of these solutions, an absorbance spectra was measured and the absorbance at 446 nm (λ_{max} , wavelength of maximal absorbance for acid form) and 616 nm (λ_{max} for base form) was determined. The ratio (Re) of absorbance for deprotonated form (at 616 nm) to absorbance for protonated form (at 446 nm) was calculated for all solutions. Standard curves were prepared by plotting Re vs. pH and log Re vs. pH (Figure 5).

A standard curve at $\text{pH}^* 3.00$ was established using formate buffer and 2,6-dinitrophenol as the indicator (Figure 6). The experimental protocol used was the same as described above. The absorbance at λ_{max} for acidic and basic forms were found to be at 348 nm and 430 nm respectively.

2.6 DETERMINATION OF pH^* IN AQUEOUS / ORGANIC SOLUTIONS

The molarity of the pure ethanol, 2-methyl-2-propanol, 2-methyl-1-propanol, and 2-butanol were calculated using the respective densities and molecular weights. An appropriate volume of aqueous / organic stock solution in 0.05M acetic acid (for $\text{pH}^* 4.0$) at 90% of the maximum

Figure 5: Aqueous standard curve at pH 3.00. Plot of log Re versus pH with 2,6-dinitrophenol as the indicator.

Figure 6: Aqueous standard curve at pH 4.00. Plot of log Re versus pH with bromocresol green as the indicator.

concentration was prepared for each alcohol. A 1990 μL aliquot of the aqueous / organic stock solution was placed in a spectrophotometer cell and scanned as the blank. A 10 μL aliquot of the stock indicator solution was then added to the alcohol solution. After mixing thoroughly, the solution was scanned, and the absorbance at λ_{max} for the acidic and basic forms of the indicator were obtained and used to calculate the R_e value. Based on the results from the aqueous standard curve, either 1 M HCl, or 1 M NaOH was added to obtain desired R_e value corresponding to the desired pH^* . The pH of the solution was then measured directly with a pH meter (pH^*). The pH^* for the remaining aqueous / organic stock solution was adjusted to the desired value by adding the smallest amount of acid or base until the pH^* was the same.

Stock aqueous / organic solutions were prepared at $\text{pH}^* 4.00$ as mentioned above. A dilution series was prepared by mixing appropriate volumes of these solutions in 1 M increments. A 1990 μL aliquot of each was taken and delivered into a spectrophotometric cell and scanned as the blank. A 10 μL aliquot of the indicator solution was added and mixed thoroughly using hand held mixer. Then the solution was scanned and the absorbance at the λ_{max} for both the acidic and basic forms of the indicator were determined to calculate the R_e value. Based on the results from the aqueous standard curve, either 1 M HCl, or 1 M NaOH was added to each solution to obtain the desired R_e value corresponding to the desired pH^* . The pH of each solution was then measured directly with a pH meter (pH^*). The results were examined by plotting initial pH^* vs. M denaturant and final pH^* vs. M denaturant. For ethanol and 2-methyl-

2-propanol the initial pH^* vs. Molar denaturant plot was found to be nonlinear, indicating that the solutions in the dilution series must be adjusted individually. The pH^* vs. M denaturant plot gave the pH^* value to which the unit molar solutions must be adjusted. Similar experiments were performed for $\text{pH}^* 3$ and similar results were obtained.

2.7 PREPARATION OF DENATURANT DILUTION SERIES

Each denaturant dilution series was prepared from the highest concentration of denaturant stock solution and corresponding aqueous buffer solution as noted above. The high molar denaturant solutions were prepared and the pH^* was adjusted to the appropriate value according to the standard curve. The initial concentration was chosen such that the final concentration of the denaturant, after the addition of the aqueous protein stock solution, would be a concentration that would correspond to unit molar value. Each set of denaturants was prepared to a total volume of 16 mL by mixing appropriate volumes of aqueous buffer and high concentration alcohol solution. All the solutions in the denaturant series were adjusted individually to the appropriate pH^* using results from plot pH^* vs. M denaturant as a guide. Half-molar concentration solutions were prepared by mixing 4 mL of adjacent unit molar concentrations. Three clean, labeled test tubes were prepared for each alcohol concentration. One test tube was for the blank and the other two test tubes were for the duplicate samples. A 1990 μL aliquot of each concentration was pipetted into individual labeled test tubes. An aliquot of 100 μL of concentrated protein stock solution (5 mg/mL) was added to two of the sample test

tubes and an aliquot of 100 μL of the aqueous buffer was added to the third test tube to serve as a blank. The final concentration of the protein in each test tube was approximately 18 μM . Each test tube was stoppered with a polypropylene stopper and mixed by inverting the test tube several times followed by vigorous vortex mixing. The solutions were allowed to equilibrate at room temperature for 30 minutes to allow the protein to unfold to an equilibrium population of molecules. The same solutions were used for fluorescence and CD measurements.

2.8 FLUORESCENCE-MONITORED CHEMICALLY-INDUCED DENATURATION TRANSITIONS

All the fluorescence experiments were performed on Spex Fluorolog DM 3000. The cuvet cooling block was equilibrated to 17.0 ± 0.2 $^{\circ}\text{C}$. Temperature was monitored with a digital thermometer by placing the probe into a cuvet located in the cooling block. The excitation and emission wavelengths were set to 280 nm and 305 nm respectively. The solutions were pre-cooled in a chilled water bath to a temperature of 17.0 ± 0.2 $^{\circ}\text{C}$ prior to taking the measurements. The blank values were auto-zeroed for all the denaturants over the entire concentration range. The measurements were taken in increasing order of denaturant concentration. Replicate solutions were measured back to back. After each measurement, the sample was removed with a pasteur pipette and stored for the CD measurements.

To examine the extent of unfolding for a set of solutions, spectral properties of the protein unfolded in 6 M guanidine hydrochloride solution

were measured to serve as a standard. It is well established that this concentration of the denaturant guanidine hydrochloride completely denatures the RNase A (Santoro and Bolen, 1988). Guanidine hydrochloride solutions were prepared by dissolving appropriate amount of guanidine hydrochloride crystals in deionized distilled water. A 1990 μL aliquot of 6 M guanidine hydrochloride solution was pipetted into each three clean labeled test tubes. A 100 μL of the concentrated protein stock solution was added to two of the three test tubes and 100 μL of the aqueous buffer was added to the third to serve as a blank. All the three test tubes were stoppered and well mixed by inverting the test tube several times, followed by vortex mixing. The solutions were allowed to equilibrate at room temperature for 30 minutes before any measurements were taken.

2.9 CD-MONITORED CHEMICALLY-INDUCED DENATURATION TRANSITIONS

The far UV-CD measurements were carried out on a Cary 61 spectropolarimeter. All the measurements were carried out with 1.00 mm path length cylindrical CD cell. During all the measurements the instrument was purged with nitrogen and the temperature held constant at 17 ± 0.1 °C. The CD measurements for each set of conditions were performed with the same solutions that were used for the corresponding fluorescence measurements. After temperature equilibration, each solution was scanned on the 0.02 degree scale from 260 nm to the lowest possible wavelength. Replicate solutions were scanned in a back to back fashion.

The blank solutions were scanned and the results were subtracted from the appropriate sample spectra. The ellipticity at 222 nm, which corresponds primarily to helix content, was taken for each set of solvent conditions and analyzed.

2.10 DATA ANALYSIS

In general, for these type of folding experiments, the data is analyzed by two different methods, the linear extrapolation method and the least-squares fitting method. These types of data analysis require complete denaturation curves including well defined pre and posttransition baselines. But in our experiments, a posttransition baseline could not be obtained, as the transition region was very close to the maximum concentration of alcohols examined. For this reason, the raw data was first converted into percent protein denatured, using RNase A in 6 M guanidine hydrochloride as a standard for denatured protein. The standard serves as a substitute for the posttransition base lines. The conversion of raw amplitude data (F_{exp}) from CD and fluorescence data to the percent denatured protein molecules present was accomplished by the following relation:

$$\% \text{ Denatured protein} = 100 \times (F_{\text{exp}} - F_{\text{N}}) / (F_{\text{U}} - F_{\text{N}})$$

where F_{exp} is the raw data for fluorescence or CD amplitude observed at a particular concentration, F_{U} is the fluorescence or CD amplitude value obtained for RNase A in 6 M guanide hydrochloride and F_{N} is the

fluorescence or CD amplitude value obtained for RNase A in 0 M denaturant. The percent denatured protein as a function of alcohol concentration was plotted and the transition midpoint (C_m , where 50 % of protein is denatured), was estimated by inspection.

The value of C_m is useful for comparing the stability of a protein under different experimental conditions. The lower the C_m , the lower the stability of the protein under the particular experimental conditions.

3.0 RESULTS AND DISCUSSION

3.1 PROTONIC ACTIVITY FOR AQUEOUS / ALCOHOL SOLUTIONS BY THE INDICATOR METHOD

It is well established that the stability of globular proteins is pH dependent. Hence, in folding experiments, the protonic activity of all the solutions should be identical. For this reason, we adapted the indicator method described by Douzou (1977) to obtain the desired protonic activity.

Aqueous / alcohol solutions were prepared in unit molar increments by mixing , appropriate volumes of aqueous and concentrated alcohol solutions, from stock solutions of correct protonic activity. Using the R_e value obtained from the absorbance spectra the protonic activity of each solution was determined. It was found that the protonic activity of all the solvent systems differed significantly from the desired value. Hence, each solution was adjusted to correct protonic activity individually. This protonic activity correction was achieved by developing a pH^* Vs. M alcohol standard curve for each set of experimental conditions (Figures 7 and 8). Individual unit molar alcohol solutions containing appropriate indicator were adjusted to the desired paH^* using spectrophotometric method as outlined above. The pH^* was measured directly with the pH meter. pH^* vs. M alcohol was plotted for ethanol and 2-methyl-2-propanol for both paH^* 3 and 4 and are shown in Figures 7 and 8. From the plots of pH^* vs. M alcohol, the pH^* value to which the individual

Figure 7: Final pH* versus Molar concentration of Ethanol. Plot of pH* versus molarity of ethanol for pH* 3.00 and 4.00. The triangles (▲) represent the values obtained at pH* 3.00 and the squares (■) represent the values obtained at pH* 4.00, using 2,6-dinitrophenol and bromocresol green as the indicators respectively.

Figure 8: Final pH* versus Molar concentration of t-butyl alcohol. Plot of pH* versus molarity of t-butyl alcohol for paH* 3.00 and 4.00. The triangles (▲) represent the values obtained at paH* 3.00 and the squares (■) represent the values obtained at paH* 4.00, using 2,6-dinitrophenol and bromocresol green as the indicators respectively.

unit molar solutions must be adjusted to obtain the desired pH^* were obtained.

3.2 DENATURATION OF RNASE A BY MONOHYDRIC ALCOHOLS

The concentration-mediated denaturation of RNase A was examined with the monohydric alcohols ethanol and 2-methyl-2-propanol. The unfolding transitions were monitored by both fluorescence and far UV circular dichroism at 17 ± 0.2 °C with a constant protonic activity of 3.00 and 4.00 using 0.05 formate and acetate buffers respectively. For each particular set of conditions, both fluorescence and CD measurements were carried out using the same solutions. All the fluorescence measurements were done with the excitation and emission wavelengths of 280 nm and 305 nm respectively. CD spectra were measured from 260 nm to 218 nm and changes in the far UV-CD signal at 222 nm was used to characterize the changes in the α -helix content of the protein. Attempts were made to examine the denaturation of RNase A with monohydric alcohols 2-methyl-1-propanol and 2-butanol. But both these alcohols have low solubility in water. Further, RNase A was found to be stable in the highest soluble concentration of these two alcohols. Hence, 2-methyl-1-propanol and 2-butanol were not used to study the denaturation transition of RNase A.

3.2.1 Denaturation of RNase A by Ethanol

Denaturation of RNase A by ethanol at pH^* 3.00 and at constant temperature of 17 °C was monitored by both fluorescence spectroscopy and circular dichroism. The denaturation curves are shown in Figure 9.

Figure 9: Fluorescence and CD data for the ethanol-mediated denaturation of RNase A at pH* 3.00. Percent denatured protein obtained from the denaturation of RNase A (18 μ M) at pH* 3.00, 0.05 M formate buffer and 17 $^{\circ}$ C, as monitored by intrinsic fluorescence (-▲- 280/305 nm) and circular dichroism (CD -■- 222 nm). The negative scale for CD data is due to the increase in the amplitude of the CD signal at 222 nm indicating an increase in the secondary structure.

An increase in the fluorescence was observed with increasing alcohol concentration, indicating the loss of tertiary structure. A post transition baseline could not be obtained, as at the higher concentration of alcohol protein aggregation was observed. In order to quantify the extent of the unfolding observed, the data was compared to the fluorescence emission value obtained from RNase A denatured in 6 M guanidinium chloride. Interestingly, the fluorescence emission of RNase A in 14 M ethanol was found to be higher than that of 6 M guanidinium chloride. In other words, the fluorescence emission of RNase A in 14 M ethanol is greater than that for totally unfolded protein. This increase in fluorescence emission of RNase A in ethanol could be due to the solvent effect where ethanol may be enhancing the fluorescence emission of tyrosine residues in the protein.

To examine this solvent effect, the following experiment was done using N-acetyl-L-Tyrosine amide as a standard for tyrosine residues in the protein. A constant amount of N-acetyl-L-Tyrosine amide (108 μ M, which is equivalent to six times the protein concentration used in the experiment) was added to the solutions of increasing concentrations of ethanol. After mixing thoroughly, fluorescence measurements were done with excitation and emission wavelengths of 280 nm and 305 nm respectively. An increase in the fluorescence was observed with an increasing concentration of ethanol (Figure: 10), indicating that as the solvent (ethanol) concentration is increased, the fluorescence emission of N-acetyl-L-tyrosine amide also increases.

The results of the above experiment clearly show that the fluorescence properties of the tyrosines in RNase A increase linearly with

Figure 10: Fluorescence of N-acetyl tyrosine amide in ethanol. N-acetyl tyrosine ethyl amide ($108\text{ }\mu\text{M}$) with increasing concentration of ethanol.

increasing ethanol concentration. This indicates that one cannot use the fluorescence emission value obtained from RNase A denatured in 6M guanidinium chloride to quantify the extent of unfolding observed under these conditions. Hence, in order to quantify the extent of unfolding observed, the data was compared to the fluorescence emission value obtained from RNase A denatured in 5 M guanidinium chloride / 14 M ethanol (it is assumed that RNase A is fully denatured in this). Using this standard, the percent denaturation data was obtained. The results from the denaturation curves show that approximately fifty percent of denaturation was observed. The transition midpoint was estimated for the fluorescence data, as the observed transition was not complete. The estimated transition midpoint is around 15 M.

The unfolding transition of RNase A at pH* 3.00 was also observed by far UV-CD by measuring the ellipticity at 222 nm. The percent denaturation data (Figure 9) shows an unexpected increase in the CD signal, which is an increase in the amplitude of CD signal at 222 nm. This amplitude increase indicates that there is an increase in the secondary structure, and in particular helix. Comparison with fluorescence data indicates that the loss of tertiary structure is accompanied by an increase in secondary structure. The transition midpoint for the CD data was not calculated, as no comparative standard is available.

At pH* 4.00, ethanol denaturation was limited to concentrations lower than 14 M, as at higher concentrations protein precipitation was observed. The fluorescence data for ethanol-mediated denaturation at pH* 4.00 is shown in Figure 11. In contrast to the ethanol-mediated

Figure 11: Fluorescence and CD data for the ethanol-mediated denaturation of RNase A at pH* 4.00. Percent denatured protein obtained from the denaturation of RNase A (18 μ M) at pH* 4.00, 0.05 M acetate buffer and 17 $^{\circ}$ C as monitored by intrinsic fluorescence ($\blacktriangle$ - 280/305 nm) and circular dichroism (CD $\blacksquare$ - 222 nm). The negative scale for CD data is due to the increase in the amplitude of the CD signal at 222 nm, indicating an increase in the secondary structure.

denaturation at $\text{pH}^* 3.00$, the denaturation data at $\text{pH}^* 4.00$ shows a small increase in fluorescence emission (Figure 11). The total unfolding transition was not observed due to protein aggregation at the higher alcohol concentration. The unfolding transition of RNase A at $\text{pH}^* 4.00$ was also observed by far UV-CD by measuring the ellipticity at 222 nm. The data (Figure 11) shows a small increase in the CD signal at 222 nm. This indicates that there is a small increase in the secondary structure and, in particular, helix. Comparison with fluorescence data indicates that small loss of tertiary structure is accompanied by small increase in secondary structure. The transition midpoint for the CD data was not calculated, as no comparative standard is available.

3.2.2 Denaturation of RNase A by Tertiary Butyl Alcohol

Figure 12 shows the tertiary butyl alcohol-mediated denaturation profiles for RNase A at $\text{pH}^* 3.00$ as monitored by intrinsic fluorescence and far UV-CD at 17°C. For t-butyl alcohol-mediated denaturation, the relative fluorescence increases with an increase in alcohol concentration to approximately 5 M. Alcohol concentrations above 5 M gave scattered and nonreproducible data. The results indicate that at the lower concentration of alcohol (< 5 M), the loss of tertiary structure is observed, but at higher concentrations, the protein is probably forming soluble aggregates, giving scattered data. A posttransition baseline could not be obtained for this reason. To quantify the extent of unfolding observed, the data was

Figure 12: Fluorescence and CD data for the t-butyl alcohol-mediated denaturation of RNase A at pH* 3.00. Percent denatured protein obtained from the denaturation of RNase A (18 μ M) at pH* 3.00, 0.05 M formate buffer and 17 $^{\circ}$ C as monitored by intrinsic fluorescence (-▲- 280/305 nm) and circular dichroism (CD -■- 222 nm). The negative scale for CD data is due to the increase in the amplitude of the CD signal at 222 nm indicating an increase in the secondary structure.

compared to the fluorescence value obtained for RNase A denatured in 6 M guanidinium chloride. For t-butyl alcohol, the solvent effect on fluorescence of denatured RNase A was not studied. As the concentration range studied for t-butyl alcohol is very small, the assumption was made that for the concentration range studied, any solvent effect would be very small and can be neglected. The results show that about fifty percent of denaturation is observed. Since complete transition could not be observed, the transition midpoint was estimated from the raw data and found to be around 4 M.

The t-butyl alcohol-mediated denaturation at pH* 3.00 as monitored by far UV-CD at 17 °C are shown in Figure 12. For t-butyl alcohol mediated denaturation, the CD amplitude at 222 nm increases with an increase in alcohol concentration to approximately 5 M. The alcohol concentration above 5 M gave scattered signal as observed in the fluorescence experiments. The results indicate that at the lower concentration of alcohol (<5 M), an increase in secondary structure is observed, but at higher concentrations, the protein is probably forming soluble aggregates, giving scattered data. In order to quantify the extent of unfolding observed, the data was compared to the CD amplitude obtained from RNase A denatured in 6 M guanidinium chloride. Comparison to the fluorescence data indicates that a loss in the tertiary structure is accompanied by an increase in the secondary structure. The transition midpoint could not be estimated for CD data, as no comparative standard is available for such an increase in the secondary structure content.

At pH^* 4.00, t-butyl alcohol mediated denaturation was limited to alcohol concentrations below 6.5-7 M, as protein precipitation was observed at higher concentrations of the alcohol. The fluorescence data for t-butyl alcohol-mediated denaturation at pH^* 4.00 is shown in Figure 13. In contrast to the t-butyl alcohol mediated denaturation at pH^* 3.00, the denaturation data at pH^* 4.00 shows only a small increase in fluorescence emission (Figure 13). The total unfolding transition was not observed due to the aggregation problem. The t-butyl alcohol-mediated denaturation of RNase A at pH^* 4.00 was also observed by far UV-CD by measuring the ellipticity at 222 nm. The data (Figure 13) shows no significant increase or decrease in the CD signal at 222 nm. This indicates that there is no significant change in the secondary structure of the protein even though the fluorescence data indicates a loss in tertiary structure.

3.3 TRANSITION MIDPOINTS OF THE OVERALL SERIES OF ALCOHOLS

In this laboratory, the concentration-mediated denaturation of RNase A has been examined using series of monohydric alcohols which differ incrementally in hydrophobicity. Specifically, methanol, ethanol, 1-propanol, 2-propanol, and 2-methyl-2-propanol were examined. Denaturation of RNase A by methanol, 1-propanol, and 2-propanol were presented previously by S.Subbiah (1995). The transition midpoints for the fluorescence data for all alcohols at pH^* 3.00 are summarized in Table 1. The data clearly shows that transition midpoints decrease with an increase in the chain length of the alcohol. In other words, the

Figure 13: Fluorescence and CD data for the t-butyl alcohol-mediated denaturation of RNase A at pH* 4.00. Percent denatured protein obtained from the denaturation of RNase A (18 μ M) at pH* 4.00, 0.05 M acetate buffer and 17 $^{\circ}$ C as monitored by intrinsic fluorescence (-▲- 280/305 nm) and circular dichroism (CD -■- 222 nm). The negative scale for CD data is due to the increase in the amplitude of the CD signal at 222 nm indicating an increase in the secondary structure.

Table 1: Transition Midpoints for the Fluorescence-Monitored Denaturation of RNase A at pH* 3.00

Alcohol	Transition Midpoint (M)
Methanol ^a	21
Ethanol	15
Propanols ^a	9-10
t-Butyl Alcohol	4

Transition Midpoints for Fluorescence data at pH* 3.00 were estimated from the raw data. Transition midpoints for CD data were not determined as an increase in signal was observed. Transition midpoints for both fluorescence and CD data at pH* 4.00 were not calculated, as no change in secondary or tertiary structure was observed.

^a Results from S.Subbiah's work (1995)

transition midpoint decreases with increasing hydrophobicity of the alcohol. Further, transition midpoints decrease linearly with respect to the number of carbon atoms (Figure 14). This trend in the transition midpoints is comparable to the results obtained by Schier et al., (1965). In their thermal denaturation study of RNase A using 1 M alcohol solutions, they observed a decrease in the thermal stability of the RNase A as the hydrophobicity of the alcohol increased. In other words, as the carbon number increases, the thermal transition midpoint decreases. The decrease in thermal transition temperature was found to be linear for methanol, ethanol, isopropanol and t-butyl alcohol. According to Schier, the lowering of transition temperature of RNase A with increase in chain length of alcohol is largely entropy dependent and the lowering of the transition temperature is the result of the binding of the nonpolar portion of the alcohol molecule to the denatured protein.

3.4 AGGREGATION

The aggregation observed for ethanol and t-butyl alcohol at higher concentrations was observed in the form of turbidity of protein solution. The aggregation was much greater and started with slightly lower alcohol concentration at pH^* 4.00 as compared to pH^* 3.00. The onset of aggregation began at 14 M for ethanol at pH^* 4.00 and 14.75 M at pH^* 3.00. For t-butyl alcohol, the onset of aggregation began at 7 M at pH^* 4.00 and 7.75 M at pH^* 3.00. Hence, all the fluorescence and CD measurements were limited to 13.5 M at pH^* 4.00 and 14.5 M at pH^*

Figure 14: Transition midpoints at $\text{pH}^* 3.00$ from fluorescence data.
Plot of transition midpoints (refer to Table 1.) versus number carbons for the overall series of monohydric alcohols studied.

3.00 for ethanol and 6.5 M at pH^* 4.00 and 7.25 M at pH^* 3.00 for t-butyl alcohol solutions.

The aggregation observed for all the alcohols was quite surprising, since one would expect that as the protein denatures, the exposure of its hydrophobic groups to the solvent would render it more soluble in hydrophobic solvents. Thus, one would expect that the denatured state of RNase A would be more soluble at higher alcohol concentrations than at lower alcohol concentrations. The observed aggregation at higher concentration of alcohol could be due to the fact that the dielectric constant of the solution decreases with increasing alcohol concentration. As the dielectric constant decreases, the electrostatic and dipolar forces increase. In this case, the electrostatic attraction between the protein molecules at higher concentration of alcohol may be enhanced to the extent that the hydrophobic attractive forces between the protein molecule and the alcohol molecules is smaller and protein aggregation is observed. Protein molecules are more highly charged at pH^* 3.00 than at pH^* 4.00. Hence, the aggregation at pH^* 3.00 is observed at higher alcohol concentrations than at pH^* 4.00, as the stronger electrostatic repulsion at pH^* 3.00 will destabilize the aggregate structures to a greater extent.

4.0 CONCLUSIONS

The most significant conclusion from this study is that transition midpoints decrease with an increase in the hydrophobicity of the alcohol. The C_m values obtained from this work and Subbiah's work (1995) are 21 M for methanol, 15 M for ethanol, and 9-10 M for propanols, and 4 M for t-butyl alcohol. The fluorescence data and CD data show that there is a loss in tertiary structure which is accompanied by an increase in the secondary structure. The results are consistent with Subbiah's work with methanol and propanols. The loss in tertiary structure is accompanied by an increase in secondary structure.

Denaturation studies of RNase A with all the alcohols at $\text{pH}^* 3.00$ show an interesting result in the form of an increase in secondary structure which accompanies the loss in tertiary structure. This is consistent with the formation of a molten globular state.

Denaturation studies of RNase A with all the alcohols at $\text{pH}^* 4.00$ show no significant change in either tertiary structure or secondary structure.

The transition midpoints decrease by about 5.5 M with an increase in carbon atoms, indicating the importance of hydrophobic effect for the stability of RNase A.

REFERENCES

- Baldwin, R.L. *Ann.Rev.Biochem.* **1975**, 44, 453.
- Biringer, R.G. San Jose State University, Unpublished results.
- Biringer, R.G.; Fink, A.L. *J.Mol.Biol.* **1982**, 87, 160.
- Birnger, R.G.; Fink, A.L. *J.Mol.Biol.* **1982**, 22, 381.
- Brandts, J.F.; Hunt, H. *J.Am.Chem.Soc.* **1967**, 89, 4826.
- Creighton, T.E. *J.Biochem.* **1990**, 1, 270.
- Dill, K.A.; Shortle, D. *Ann.Rev.Biochem.* **1991**, 60, 795.
- Dill, K.A.; Fiebig, K.M.; Chan, H.S. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, 90, 1942.
- Dill, K.A. *Biochemistry.* **1990**, 29, 7133.
- Douzou, P. In "*Cryobiochemistry: An Introduction*", Academic Press, **1977**; Chapter 2, pp. 45-50.
- Jaenicke, R. *Biochemistry.* **1991**, 3147, 30.
- Karplus, M.; Weaver, D.L. *Nature.* **1976**, 260: 404.
- Karplus, M.; Weaver, D.L. *Protein Science.* **1994**, 3, 650.
- Kim, P.S.; Baldwin, R.L. *Ann.Rev.Biochem.* **1982**, 51, 459.
- Kuwajima, D. *Proteins: Structure, Function, and Genetics.* **1989**, 6, 87
- Levitt, M. *J.Mol.Biol.* **1976**, 59, 104.
- Lins, L.; Brasseur, R. *FASEB J.* **1995**, 9, 535.
- Pace, C.N.; Laurents, D.V.; Thomson, J.A. *Biochemistry.* **1990**, 29, 2564.
- Privalov, P.L.; Gill, S.J. *Pure Appl. Chem.* **1989**, 61, 1097.

Ptitsyn, O.B. In *Protein Folding*: Creighton, T.E., Ed.: W.H. Freeman and Co: New York, 1992: Chapter 6, pp.243-280.

Ptitsyn, O.B. *J. Protein Chemistry*. **1987**, 6, 273.

Sage, H.J.; Singer, S. *J. Biochemistry*. **1962**, 1, 305.

Santoto, M.M.; Bolen, D. W. *Biochemistry*. **1988**, 27, 8063.

Schier, E.E.; Ingwall, R.T.; Scheraga, H.A. *J. Phy. Chem*. **1965**, 69, 298.

Shirley, B.A; Stanssens, P.; Hahn, U.; Pace, C.N. *Biochemistry*. **1992**, 31, 725.

Stryer, L. *Science*. **1968**, 162, 526.

Subbiah, S.; M.S. Thesis.

Tanford, C. *Adv. Prot. Chem*. **1968**, 23, 121.

Wetlaufer, D.B. *Proc. Natl. Acad. Sci. U.S.A*. **1973**, 70, 697.