1994

Preparation, characterization, and refolding of C[epsilon]⁷³-NH₂ ribonuclease

Ohlan Randy Brooks
San Jose State University

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

Recommended Citation

Brooks, Ohlan Randy, "Preparation, characterization, and refolding of C[epsilon]⁷³-NH₂ ribonuclease" (1994). *Master's Theses*. 823.

DOI: <https://doi.org/10.31979/etd.fwy6-fkmy>

https://scholarworks.sjsu.edu/etd_theses/823

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.

U·M·I

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

Order Number 1359006

**Preparation, characterization, and refolding of C^{ε73}-NH₂
ribonuclease**

Brooks, Ohlan Randy, M.S.

San Jose State University, 1994

U·M·I

**300 N. Zeeb Rd.
Ann Arbor, MI 48106**

PREPARATION, CHARACTERIZATION, AND REFOLDING
OF C¹³-NH₂ RIBONUCLEASE

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
Ohlan Randy Brooks
June, 1994

© 1994

Ohlan Randy Brooks

ALL RIGHTS RESERVED

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

A handwritten signature in cursive script, appearing to read "Roger G. Biringer", written over a horizontal line.

Dr. Roger G. Biringer

A handwritten signature in cursive script, appearing to read "Roy Okuda", written over a horizontal line.

Dr. Roy Okuda

A handwritten signature in cursive script, appearing to read "Joseph J. Pesek", written over a horizontal line.

Dr. Joseph J. Pesek

APPROVED FOR THE UNIVERSITY

ABSTRACT

PREPARATION, CHARACTERIZATION, AND REFOLDING OF $C^{\epsilon 73-NH_2}$ RIBONUCLEASE A

by Randy Brooks

The diazonium salt of an RNase inhibitor analog, 5'-(4-aminophenylphosphoryl) uridine was synthesized using dicyclohexylcarbodiimide (DCC) coupling. The diazonium salt was used to azotize tyrosine-73 of Ribonuclease A (RNase A) and subsequently reduced to yield the aminotyrosyl derivative $C^{\epsilon 73-NH_2}$ RNase. This derivative was purified by affinity column chromatography and characterized. Oxidation of this aminotyrosyl derivative was attempted by various means to produce the corresponding nitrotyrosyl derivative and also to insure stability of the amino compound during folding experiments. The refolding of the aminotyrosyl derivative was monitored at pH 3.0 and pH 6.0 in 35% methanol and $-15^{\circ}C$. Kinetics were evaluated from the refolding data and compared to previous experiments under the same experimental conditions.

ACKNOWLEDGEMENT

I would like to thank Dr. Biringer for his guidance, encouragement and support throughout my graduate research. In addition, I would like to thank Dr. Pesek and Dr. Okuda for their suggestions on my thesis.

TABLE OF CONTENTS

	Page
Abstract	iii
Acknowledgement	iv
List of Tables.	vi
List of Figures.	vii
List of Abbreviations	ix
INTRODUCTION	1
1.1 Protein Folding.	1
1.2 Driving Force for Protein Folding	3
1.3 Models for Protein Folding	6
1.4 Measurement of Protein Folding	11
1.5 RNase A as a Model for Protein Folding	20
MATERIALS AND METHODS	32
2.1 Synthesis of 5'-(azophenyl phosphoryl)-uridine.	32
2.2 Synthesis of C ^ε 73-NH ₂ RNase	36
2.3 Characterization of C ^ε 73-NH ₂ RNase ...	39
2.4 Refolding of C ^ε 73-NH ₂ RNase	40
RESULTS AND DISCUSSION	43
3.1 Synthesis and Characterization of C ^ε 73-NH ₂ RNase.	43
3.2 Characterization of C ^ε 73-NH ₂ RNase	59
3.3 Folding of C ^ε 73-NH ₂ RNase	67
REFERENCES	87

LIST OF TABLES

Table	Page
1. Kinetics of Refolding of RNase A and Derivatives	27
2. Kinetic Data for Refolding of C ^ε 73-NH ₂ RNase	84

LIST OF FIGURES

Figures	Page
1. Typical Denaturation Curve	17
2. Scheme 1: DCC coupling of 5'UMP and PNP.	45
Scheme 2: Reduction of PNPU to PAPU.	46
Scheme 3: Azotization of PAPU	47
3. Chromatogram of DCC Reaction Mixture.	50
4. UV Spectrum of PNP.	51
5. Chromatogram of EDC Reaction Mixture.	53
6. UV Spectrum of PAPU	54
7. Chromatogram of Diazotization Reaction Mixture.. . . .	56
8. UV Spectrum of Azo-RNase	57
9. Chromatogram of Reduced Azo- RNase Reaction Mixture . .	58
10. Affinity Column Purification	60
11. UV Spectrum of C ^ε 73-NH ₂ RNase	61
12. NMR Spectrum of Aminotyrosine	62
13. NMR Spectrum of C ^ε 73-NH ₂ RNase	63
14. Isoelectric Focusing Gel	65
15. Fluorescence of C ^ε 73-NH ₂ RNase	70
16. Fluorescence of C ^ε 73-NH ₂ RNase in 6M Guanidinium Chloride	71
17. Excitation Spectrum of C ^ε 73-NH ₂ RNase	72
18. Fluorescence of C ^ε 73-NH ₂ RNase Excitation at 342 nm	73
19. Fluorescence of Aminotyrosine at pH 6.0.	75
20. Fluorescence of Aminotyrosine at pH 3.0.	76
21. Excitation of Aminotyrosine pH 6.0 and 3.0	77

22.	Fluorescence of RNase and Amino Derivatives	78
23.	Refolding of C ^ε 73-NH ₂ RNase at pH 3.0.	80
24.	Semilog Plot at pH 3.0	81
25.	Refolding of C ^ε 73-NH ₂ RNase at pH 6.0	82
26.	Semilog Plot at pH 6.0	83

LIST OF ABBREVIATIONS

1. BPTI: Bovine Pancreatic Trypsin Inhibitor
2. C^{ε115,76,73}: Modification of tyrosine-115, tyrosine-76 and tyrosine-73 at the epsilon position
3. C^{ε115,76}: Modification of tyrosine-115 and tyrosine-76 at the epsilon position
4. C^{ε115}: Modification of tyrosine-115 at the epsilon position
5. C^{ε73}: Modification of tyrosine-73 at the epsilon position
6. DCC: Dicyclohexylcarbodiimide
7. EDC: 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
8. GdmCl: Guanidinium chloride
9. IEF: Isoelectric Focusing
10. I_N: Native-like intermediate
11. N: Native State of RNase A
12. NMR: Nuclear magnetic resonance
13. PAPU: 5'(4-Aminophenyl phosphoryl)-uridine
14. PNPU: 5'(4-Nitrophenyl phosphoryl)-uridine
15. RNase A: Ribonuclease A
16. TNM: Tetranitromethane
17. U: Unfolded state of RNase A
18. U_F: Fast unfolding form of RNase A
19. UMP: Uridine 5'-monophosphate
20. U_S: Slow unfolding form of RNase A
21. UV: Ultraviolet
22. ΔG: Change in Gibbs Free Energy

INTRODUCTION

1.1 Protein Folding

Overview. Protein folding, the process by which a polypeptide chain with random configuration achieves the highly structured state of the native protein, is an area of increasing interest. In the past thirty years, researchers in biochemistry and molecular biology have gained a thorough understanding of the triplet "genetic code" by which the nucleotide sequence of nucleic acids determine the amino acid sequences of the polypeptide chains that form proteins. This "code," however, gives only a portion of the information necessary for understanding the relationship between DNA and protein. The correlation between the amino acid sequence and the folded biologically competent protein remains to be resolved.

The sequence of amino acids in a protein, the primary sequence, is ultimately responsible for the final structure of the native protein. However, no simple code has been established to relate this primary sequence to the final native state. This is because the relationship between the primary amino acid sequence and the native structure is extremely complex and involves an immense number of intermolecular and intramolecular interactions. The mechanism of protein folding has been studied by many researchers to gain information about the relationship between the primary sequence and native structure of proteins.

Numerous models have been proposed to define the process of

protein folding. Some assume that folding is the result of random fluctuations of the polypeptide chain biased by elements of structure inherent to the molecule. Others suggest that the pathway of protein folding is dominated by the hydrophobic collapse of secondary structure to form a native-like, "molten-globule" state followed by structural rearrangement to form the native state. Still, others postulate that protein folding is a sequential process governed by a series of well-defined intermediate structures.

A variety of different tools have been used to study protein folding and many different experimental methods have been developed. Analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy, visible and ultraviolet absorbance and fluorescence spectroscopy, catalytic activity and inhibitor binding (in the case of proteins that are enzymes), and circular dichroism (CD) have been used to characterize the structures along the path of protein folding. Proteins have been chemically modified to create spectroscopic probes for measuring structural changes in specific regions of the protein molecules during folding. Equilibrium studies, kinetic unfolding and refolding experiments and modifications of these techniques have all been used to gain information about the pathway for protein folding.

Bovine pancreatic ribonuclease A (RNase A) has been the subject of numerous folding studies as a result of its inherent stability, intrinsic absorbance and fluorescence probes, and well-defined structure. Partially folded intermediates have been observed in the folding of RNase A which suggests a sequential

pathway for this protein. Methanol-based cryosolvents and subzero temperatures have also been used to help stabilize and populate intermediate structures for the purpose of fully characterizing the folding pathway.

1.2 Driving Force for Protein Folding

Native Structure. To be biologically active, proteins must adopt specific three-dimensional structures upon folding. No protein is known to adopt alternative, fully-folded conformations (Creighton, 1990). The protein is unique in this sense as compared to other types of polymer molecules. The native structure is compact and highly ordered as compared to the unfolded "random" polypeptide chain from which it was formed.

The native structure is formed, ultimately, through the specific interactions of the amino acid residues in the unfolded chain. These interactions include van der Waals forces, hydrogen bonds, charge-charge attractions and repulsions, and hydrophobic interactions. The relative importance of these interactions is the center of considerable controversy. According to Creighton (1984), "within the polar aqueous environment, noncovalent interactions between different parts of the polypeptide chain, by electrostatic 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". In contrast, the work of Chen *et al.* (1993) has shown that a single hydrogen bond can make a significant

contribution to protein stability. It is generally agreed, however, that hydrophobic interactions are the most important forces that define the formation and overall stability of the native structure.

Hydrophobic Interactions. Water is a poor solvent for apolar compounds because of the absence of hydrogen bonding between solvent and solute. Because of this, water molecules must form a "highly-ordered cavity" in which the apolar solute will fit in order for solvation to occur. Although solvation is slightly favored by enthalpy, the entropy associated with the "ordering" of the water molecules is so unfavorable (at room temperature) that the associated ΔG (Gibbs free energy) for the process is positive, and solvation does not occur to any extent. This fact explains the observation that among the known structures of native proteins, polar and charged amino acid residues are posed towards the surface of the protein where they are exposed to water, whereas the nonpolar residues are usually located in the interior, shielded from the solvent. The native structure of the protein consists, then, of a hydrophobic core surrounded by polar residues. It is the coalescence of the nonpolar residues to form the hydrophobic core that serves to minimize the solvent entropy and hence contributes to the stability of the native conformation. This contribution to the stability is opposed by the unfavorable entropic effect resulting from localizing the nonpolar residues in the compact, hydrophobic interior of the protein. The difference between these two large, opposing forces is relatively small with a ΔG favoring the native state of typically -5 to -10 kcal/mole for most small proteins (Creighton, 1990).

Evidence for the Hydrophobic Mechanism. Evidence exists to support the hypothesis that hydrophobic interactions are the driving force for protein folding. It is known that nonpolar solvents denature proteins. According to the hydrophobic mechanism, the nonpolar solvent reduces the free energy of the unfolded state by solvating the exposed nonpolar amino acids and polypeptide backbone. Also, an unusual temperature dependence has been observed for proteins in which stability of the native state decreases not only at high temperatures, but also at low temperatures. The cold destabilization of proteins has been shown to resemble nonpolar solvation (Privalov and Gill, 1988).

"Spectroscopic and high-resolution differential scanning calorimetry experiments show the resemblance of the temperature dependence of the free energy of folding and the temperature dependence of the free energy of transfer of nonpolar model compounds from water into nonpolar media. Both involve large decreases in heat capacity" (Dill, 1990, p. 7151). In addition, protein stability is affected by different salt species in the same rank order as the lyotropic or Hofmeister series which is usually interpreted as the result of hydrophobic interactions.

A Balance Between Many Forces. The overall native configuration of proteins is a delicate balance between the different enthalpic and entropic consequences of hydrogen bonding, van der Waals forces, electrostatic attractions and repulsions, hydrophobic interactions and solvation effects. As a result, small changes in environmental conditions such as pH, salt concentration,

affect the stability of the native state.

The complex collection of forces that determine the native configuration of the protein must surely be reflected in protein folding and hence each interaction most certainly plays a role in the overall process. Does the native state of the protein represent the most thermodynamically stable configuration given the constraints imposed by primary structure, or does it represent the most kinetically favorable structure? Due to the extreme complexity of the protein folding process, it is not currently known whether folding is driven by thermodynamics or kinetics.

1.3 Models for Protein Folding

Early Experiments. The forces controlling the denaturation and folding of proteins and the mechanisms by which these processes occur have been subject to investigation, speculation, and controversy for over sixty years. Historically, Wu is credited with first proposing the modern view that native proteins involve regular repeated patterns that result from the folding of the polypeptide chain into a three-dimensional network held together by noncovalent forces (Dill, 1990). According to Wu, "denaturation is the breaking up of these labile linkages. Instead of being compact, the protein now becomes a diffuse structure. The surface is altered and the interior of the molecule is exposed" (Wu, 1929, p. 562). The experiments of Anson and Mirsky in 1931 and of Anson in 1945 showed that hemoglobin folding is reversible as evidenced by similarities in the solubility, crystallizability, characteristic

absorbance spectrum, binding to oxygen and carbon monoxide, and inaccessibility to trypsin digestion (Dill, 1990). It was later shown that denaturation is also thermodynamically reversible for some proteins and involves large conformational changes. Anfinsen (1973) reduced and reoxidized the disulfide bonds of RNase A to form random distributions of all possible disulfide combinations and found that the native structure and activity was regained upon refolding. Many others found that protein denaturation is reversible and cooperative under certain conditions and thus folding of small globular proteins can be described by a two-state equilibrium model involving only the native or folded state (N) and the unfolded state (U) (Schmid, 1990). However, fully cooperative two-state transitions provide no specific evidence for the mechanism of folding.

Structural and Kinetic Models. Many models for protein folding have been proposed and can be categorized into two groups: structural models and kinetic models. Structural models describe folding in terms of the structure and order of the intermediates in the folding pathway without being specific about factors that control the rate of protein folding. These models are based on x-ray and NMR data and are being refined as new and more powerful techniques are developed. Kinetic models describe the dominant intermediates and give factors that control the rate of folding without being specific about the structure of the intermediates. A number of kinetic models have been proposed to describe the mechanism of protein folding given the large amount of

experimental data.

Random Search. Levinthal (1968) first considered the possibility that proteins might fold by a purely random search of all possible configurations. However, the time required would be impossibly long (10^{50} years for a chain of 100 residues) (Kim and Baldwin, 1982), as compared to the actual observed folding time of 0.1 to 1000 seconds (Creighton, 1990). It is possible, however, that a biased random search could occur in the time frame observed for protein folding. 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 be significant in the early stages of folding.

Nucleation-Rapid-Growth. The nucleation-rapid-growth mechanism was initially considered to be one of the more promising explanations of how protein folding could occur on the appropriate time scale. In this model, a nucleation center is formed early in the folding pathway by the random conformational fluctuations of the unfolded protein. This center serves as a template upon which folding proceeds rapidly to completion. This nucleation center is unstable by itself and breaks down if not stabilized by further folding. Formation of the nucleation center is the rate-limiting step in this model and all molecules would either encounter the same center and fold by the same pathway or fold by several different pathways (Wetlaufer, 1973). Folding intermediates are not populated in this model since folding is too fast once the nucleation center is formed. This model does not account for the existence of

intermediates observed in the folding of many proteins.

Diffusion-Collision-Adhesion. The diffusion-collision-adhesion model postulates that short segments of the unfolded protein chain fold independently into compact microdomains. These microdomains are unstable by themselves but, through diffusion-driven collisions, they coalesce with other microdomains and the protein folds sequentially, one segment at a time (Karplus and Weaver, 1976). In this scheme, diffusional collision to form microdomains is considered to be the rate-limiting step. However, Kim and Baldwin (1982) pointed out that coalescence of two microdomains could also be rate limiting in certain situations.

This model was tested by refolding RNase A in solvents of different viscosities (Tsong and Baldwin, 1978). It was found that the overall folding rate was independent of solvent viscosity. Such results would not be expected if a diffusion-collision-adhesion process defines the folding process.

Molten Globule. In an attempt to explain early intermediates observed in the kinetics of protein folding, Kuwajima (1989) proposed the molten globule model. In this model, the intermediate is described as a compact globular molecule with native-like secondary structure, but unfolded tertiary structure. The rate determining step in folding involves later structural changes after the formation of the molten globule state.

Hydrophobic Zipper. Dill and coworkers (Dill *et al.*, 1993) have explained protein folding in terms of the hydrophobic zipper (HZ) model. In this scheme, hydrophobic contacts act as constraints

that bring other such contacts into spatial proximity which further constrains and "zips up" the next contacts. The HZ-heteropolymer collapse is highly cooperative and is driven by nonlocal (but topologically local) interactions. These interactions result in the formation of sheets, helices and irregular structures. Thus, "The folding process is postulated to be a fast nonexhaustive search, in which, for appropriate sequences, the early stages of H (hydrophobic) core formation are concurrent with formation of helices and sheets" (Dill *et al.*, 1993, p. 1494).

This model was tested by computer modeling for crambin and bovine pancreatic trypsin inhibitor (BPTI). Structures predicted by the model have energies within about 1.5 kcal/mole of those obtained for the actual native structures. Although this initial work shows promise, more extensive research is necessary to confirm this model.

Sequential. The sequential folding model describes folding as a sequence of unique and finite steps, similar to a metabolic pathway (Kim and Baldwin, 1982). Intermediates may be populated under suitable folding conditions. There are two variations of this model: the framework model and the modular assembly model. In the framework model, secondary structures form by hydrogen bonding early in the folding process and these structures combine to form a globular intermediate which rearranges to give the final native structure. The modular assembly variation of the model suggests that specific regions of the protein fold independently, but these regions may fold at different times. The sequential folding model predicts the existence of specific, well-populated

intermediates as observed for folding of RNase A (Cook *et al.*, 1979). Experiments performed by Schmid and Baldwin (1979) using the exchange of tritium-labeled amide protons also support the sequential model.

Conclusion. At best, experimental data lends some support to all the proposed models while also providing contradictions. Given the complexity of the nature of protein folding, it is not unlikely that the process, or more accurately, the processes that lead to the native protein from a random polypeptide chain involve elements of many of the proposed models.

1.4 Measurement of Protein Folding

Spectroscopic Probes. The first criterion for performing protein folding experiments is to establish some parameter that can be monitored and which changes as a function of the protein's structure. Over the years, a number of useful methods have been established.

One of the simplest methods is to monitor the change in UV absorbance for tyrosine and tryptophan residues. These aromatic amino acids can be used as "probes" of protein structure because the absorption bands for these residues are sensitive to changes in the local environment. Absorbance values measured at a given point in the absorption band will be different when the residues are in the hydrophobic core of the native protein as opposed to when they are exposed to the polar solvent (water) in the unfolded state. For a

typical experiment, the absorbance values for the completely unfolded protein and the native protein are first measured to establish the magnitude of the absorbance change (amplitude) that can be observed for the folding experiment. At any point in the experiment, the absorbance value as compared to the total absorbance change for the process can be used to calculate the percent folded protein present. In a similar fashion, the aromatic amino acids can also serve as fluorescence probes. The quantum yield of these residues decrease as the protein folds as a result of quenching by other residues in the protein core. Used as fluorescence probes, these residues are more sensitive to changes in the environment than the corresponding absorbance probes due to the longer lifetimes of the excited states. Consequently, intrinsic fluorescence can serve to monitor smaller changes in structure than can be observed by absorbance measurements.

Chemical Modifications. Absorbance and fluorescence determinations of protein folding using indigenous aromatic residues are limited, as these measurements represent the sum of the changes about each probe and thus give only a global picture of the process. Although this information is useful, more detailed data can be obtained by placing spectroscopic probes into specific regions of the protein. Experimental data obtained from each probe can be used for evaluating structural changes that occur during the folding of the region about the probe.

Spectroscopic probes can be created by chemically modifying amino acid side chains in the protein. Garel and Baldwin (1975),

Biringer and Fink (1988a), and Puntambaeker (1992) created spectroscopic probes in RNase A by nitrating or aminating the tyrosine residues.

Tetranitromethane can be used to selectively nitrate three of the six tyrosines in the native protein. Tyrosine (Tyr) residues 115, 76, and 73 are considered to be solvent "exposed" as compared to tyrosine residues 92, 97, and 25, as determined by x-ray crystallography and their relative reactivity to chemical modifying agents. The order of reactivity is Tyrosine-115 (Tyr-115) > Tyrosine-76 (Tyr-76) > Tyrosine-73 (Tyr-73). Reaction of RNase A with tetranitromethane under mild conditions produces a mixture of $C^{\epsilon 115}\text{-NO}_2$ RNase, $C^{\epsilon 115, 76}\text{-NO}_2$ RNase, and $C^{\epsilon 115, 76, 73}\text{-NO}_2$ RNase. The relative yield of each product is a function of reaction conditions. Control of reaction conditions and subsequent separations yield the singly, doubly, and triply nitrated derivatives. Each of these derivatives can be examined to obtain information about folding in the regions adjacent to these residues.

Biringer and Fink (1988a) examined the folding kinetics for each of these derivatives. The folding about Tyr-115 was obtained directly and data for the Tyr-76 and Tyr-73 residues were obtained by subtraction of the different data sets (Biringer and Fink, 1988a; Puntambaeker, 1992).

Seagle and Cowgill (1976) and Puntambaeker (1992) prepared these nitrate derivatives and then reduced them to the corresponding amino derivatives. The resulting aminotyrosyl derivatives give a fluorescence emission around 320-400 nm (depending on the derivative), whereas the fluorescence of the native tyrosines show a

maximum at 305 nm. Hence, this allows independent monitoring of modified and unmodified residues in folding experiments. Wilchek and Gorecki (1969, 1978) developed a novel procedure for the specific amination of Tyr-73 in RNase A using a diazonium derivative of 5'-(4-aminophenylphosphoryl)-uridine-2'(3')-diphosphate prepared from the competitive inhibitor uridine-2'(3'), 5'-diphosphate. When this inhibitor derivative was introduced into the active site of RNase A, the phenolic "spacer arm" positioned the diazonium group to couple selectively with the Tyr-73. Reduction of the resulting diazo bond and removal of the inhibitor derivative from the active site produced C^ε73-NH₂ RNase.

Tritium Exchange, NMR and Catalytic Activity. A variety of other methods have been used to monitor protein folding. Schmid and Baldwin (1979) labeled the exchangeable amide "backbone" protons in RNase A with tritium by incubation with tritium oxide (³H₂O). They first initiated refolding and then added tritium oxide at various times. They chose refolding conditions so that rapid exchange-out would occur unless folding intermediates were formed that could protect some of the protons from exchange. Thus, unexchanged protons were found in regions that became native-like before addition of tritium oxide. This technique allowed them to detect early intermediates in the folding pathway.

Nuclear magnetic resonance (NMR) spectroscopy has proven to be a powerful tool for monitoring protein folding as well as elucidating protein structure. Blum *et al.* (1978) and Biringer and Fink (1982a) 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 were able to confirm the existence of partially folded intermediates. Catalytic activity and inhibitor binding have also been used as probes to monitor the folding of enzymes. Liu and Tsou (1987) reported that RNase A loses almost all enzymatic activity in 2-3 M guanidine chloride where only slight conformation changes are detected by tyrosine fluorescence and absorbance. In addition, they found that the kinetics of inactivation are completed within the dead time of mixing.

Equilibrium Experiments. The reversible unfolding of small, single domain proteins is usually observed as a cooperative process in which only the fully native and fully unfolded species are present at equilibrium. These cases are adequately described by a two-state model involving only the folded and unfolded states. The equilibrium can be shifted by changing environmental conditions to thermodynamically favor the folded or unfolded state. This is accomplished by altering the temperature, pressure, pH, solvent or by introducing denaturants such as urea or guanidinium chloride. Equilibrium unfolding experiments are performed by taking native protein and gradually changing conditions (such as addition of denaturants, elevation of temperature, etc.) to favor the unfolded state. Assuming a simple two-state process, the equilibrium

constant can be defined as:

$$K_{eq} = [U]/[N]$$

where U is the unfolded state and N is the native state. With mild changes in conditions, little change to the native structure occurs and the native state predominates. As conditions are further changed, the transition region is reached where the equilibrium constant is near unity and both the folded and unfolded states coexist. As the conditions are changed further to favor the unfolded state, the equilibrium constant increases and the unfolded state predominates. Figure 1 shows a typical two-state folding transition. The solid line represents the fraction of unfolded molecules (f_u , right scale from 0 to 1) that was observed experimentally. Within the transition region, 2.8 to 3.2 M guanidinium chloride, the free energy difference (ΔG) between the native and unfolded states usually varies in a linear fashion with denaturant concentration. If it is assumed that change in free energy is linear over all concentrations of denaturant, free energy values at concentrations outside the transition region can be obtained. Thus, extrapolation of the free energy values to zero denaturant concentration (dashed line in Figure 1) gives the ΔG for the process in the absence of denaturant. Note that the scales for f_u

FIGURE 1: Typical denaturation curve. Solid line gives data as the fraction of unfolded molecules (fu) versus guanidinium chloride (GdmCl) concentration. The dotted line gives the change in free energy (ΔG) and the log of the equilibrium constant (K_{eq}) versus GdmCl.

and ΔG are co-linear at the midpoint of the transition region where the 0 and 1 values for f_u occur on the ΔG scale at $-2RT$ and $2RT$ respectively (Creighton, 1984).

Denaturation curves obtained using different structural probes under the same denaturing conditions should be coincidental if the process is two-state. Noncoincidental curves indicates that the process is not cooperative and two-state and that intermediate(s) are present at equilibrium.

Kinetic Measurements. The kinetics of protein refolding are examined by first unfolding the protein with heat, denaturant, or by other means and then rapidly changing the experimental conditions to favor the native state. Folding is followed by monitoring changes in the values for structural probes 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 unfolded intermediates do not accumulate to any substantial levels. If the folding/unfolding amplitude accounts for the entire signal change observed in the corresponding equilibrium transition (the difference in the signal for the folded minus the unfolded), then no partial folding/unfolding has preceded the rate-limiting step (Schmid, 1992).

The kinetics of protein refolding are often not as straight forward. If the population of the unfolded protein structure is heterogeneous, multiphasic kinetics may be observed as the sum of

multiple single exponential processes, a series of different processes in a sequential pathway, or a combination of both. If the kinetics associated with the folding to the final state involve a lag phase, then the process must be sequentially coupled to an earlier process. In such cases, all the steps preceding this step are rapid and not observed directly. In addition, it has been observed that the rate of folding depends upon the final folding conditions and not upon the nature of the initial unfolded state. "This implies that there is a rapid conformational equilibration of all the molecules prior to the rate limiting step, so that the same populations of unfolded molecules are obtained rapidly upon transfer to the same refolding conditions" (Creighton, 1984, p.145).

Modified Experiments. To obtain more information about the folding pathway, equilibrium and kinetic studies are often modified. As an example, Biringer and Fink (1982a) used methanolic cosolvents and low temperatures in refolding experiments to examine the folding pathway of RNase A. Their reasoning was as follows: "If the early stages of folding involve predominantly secondary structure, then low temperatures, which strengthen hydrogen bonds, would be expected to stabilize such early intermediate states. If the later stages of folding involve the packing together of regions of secondary structure, predominantly through hydrophobic interactions, those would be expected to be weakened at lower temperatures. Thus low temperatures should increase the discrimination between the stability of early and late folding intermediates, thus making the former more readily detectable"

(Biringer and Fink, 1988a, p.305). Using this methodology, they observed transient intermediates in the folding pathway of RNase A not normally detected under other experimental conditions.

1.5 RNase A as a Model for Protein Folding

Properties of 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 (115, 76, and 73) are considered to be "exposed" to solvent in the native structure and three tyrosines are located in the hydrophobic core of the protein as determined by X-ray crystallography and reactivity towards certain modifying agents. The peptide bonds associated with Pro-114 and Pro-93 are cis in the native conformation and isomerize to yield an equilibrium mixture of cis/trans forms in the unfolded state. The enzyme is known to undergo reversible unfolding without loss of catalytic activity. The solubility in water is quite high and the protein tends not to aggregate even in the unfolded state. As a result, RNase A has served as a model compound for many experimenters investigating the mechanism of protein folding.

Proline Isomerization. In 1973, Garel and Baldwin observed biphasic kinetics in the refolding of RNase A using the absorbance of tyrosine at 286 nm (ΔA_{286}), and the binding of the competitive inhibitor 2'-cytosine monophosphate (2'-CMP) as probes and

discovered the existence of both a fast (U_F) and a slow (U_S) folding species that refold to give the native protein. U_F was found to fold at a rate approximately 100 times faster than U_S .

Garel and Baldwin (1975) synthesized the trinitrotyrosyl (see 1.4 Measurement of Protein Folding) derivative of the protein and determined the pK values for the nitrotyrosyl residues by spectrophotometric titration. Since the pK values are sensitive to local environment, they reflect the structure of the protein about the nitrotyrosyl residues. The pK measurements of the nitrotyrosyl groups of the fast and slow folding forms were found to be different suggesting that they had different structures and were physically different species.

Brandts *et al.* (1975) proposed that the existence of proline residues in the wrong isomeric configuration would slow down protein folding, as an energy barrier of approximately 20 kcal/mol separates the cis and trans isomers in model compounds. It was postulated that in the unfolded state of the protein, the proline residues occur with both the cis and trans isomeric forms. Only those unfolded molecules in which all the proline residues have the same isomeric form as the native protein would fold directly to the native conformation. Unfolded molecules with one or more prolines in the incorrect isomeric form would fold only after first undergoing slow isomerization to an unfolded form with the same proline isomers as in the native conformation. These results led to a simple mechanism for folding of RNase A:

Schmid and Baldwin (1979) developed an assay (see 1.4 Measurement of Protein Folding) for determining the relative amount of U_F and U_S in unfolded RNase A. U_S was shown to comprise approximately 70-80% of reversibly-unfolded protein and U_F , approximately 20-30%. Studies by Schmid and Baldwin (1978) suggest that not all proline residues are essential, and these non-essential residues can isomerize after folding had occurred. The implication of this work is that events other than proline isomerization could be contributing to the slow folding forms observed.

Lin and Brandts (1983) discovered that trypsin cleaves the Lys-X-Pro bond in a peptide chain only when the proline is in the trans configuration. Using this information, they examined the isomeric configuration of the Tyr-92-Pro-93 bond in oxidized RNase A. They observed that the proline was 100% cis in the native state and 33% cis in the reversibly unfolded protein. This result supported their earlier proposal that the 70-80% slow folding protein was due to proline isomerization and showed that Pro-93 is involved.

Partially Folded Intermediates. Whenever more than one kinetic phase is observed, at least three species must be involved in the reaction (Kim and Baldwin, 1982). Brandts explained the biphasic kinetics of RNase A folding by the following sequential scheme:

In this scheme, the biphasic kinetics result from the conversion of U_S into U_F which then folds rapidly to form native protein. This scheme does not require the existence of partially folded intermediates. An alternate explanation of the same data is described by the parallel scheme:

In this scheme, 20% of the unfolded protein folds rapidly to the native form and the remaining 80%, yields the native structure through a slower process. The slow folding pathway in this scheme could be populated by partially folded intermediates.

Blum *et al.* (1978) found evidence for a structural intermediate in the early stage of RNase A refolding using the time-dependent changes in the magnitude of the C-2 resonances for histidine protons. Schmid and Baldwin (1979) detected an early hydrogen-bonded intermediate during folding at low temperatures by amide proton exchange competition experiments. This early structural intermediate is present before a compact globular structure is formed as determined by change in tyrosine absorbance. Cook *et al.* (1979) developed a specific assay to follow the isomerization of proline residues during refolding of U_S . They were able to show that extensive folding of U_S to an intermediate occurs before proline isomerization. This folded intermediate (I_N) has a tyrosine absorbance close to that of native RNase A and a binding

site for the specific inhibitor, 2'CMP.

Schmid (1980) studied the refolding of U_S under strongly native conditions (pH 6, 0.2 M guanidinium chloride, 0.2 M ammonium sulfate, and 5°C) and found that U_S consists of a major faster phase (U^{II}_S) and a minor slower phase (U^I_S). They observed that the $U^{II}_S \rightarrow N$ transition is polyphasic with different rate limiting steps, depending on whether the folding was measured by tyrosine absorption or fluorescence. Furthermore, the final species formed in this process, as measured by tyrosine absorption, is not the native enzyme, but an intermediate state, I_N . This intermediate still undergoes major changes in tyrosine fluorescence before reaching the final native state. Fluorescence detected folding of U^{II}_S has the properties expected from proline isomerization (pH independence between 2.5 and 6, rate independent of guanidinium chloride, and activation enthalpy of 73 kJ/mol). These combined results strongly suggest that the refolding of RNase A is the result a series of parallel reactions involving a hydrogen-bonded (I_1) and native-like intermediate as illustrated by the following scheme:

Multiple Unfolded State Versus Structural Intermediates. In order to account for the multiphasic kinetics observed for RNase A

folding, Lin and Brandts (1983) proposed a simple kinetic model in which multiple unfolded forms of the protein fold directly to a single native state. This model does not require structural intermediates to explain the process of protein folding. However, this hypothesis contrasts sharply with the growing body of experimental evidence which indicates that folding is a sequential process involving structural intermediates. This evidence includes (1) the observation of complex refolding kinetics, (2) the fact that substantial parts of the refolding amplitudes were missing (due to fast steps) and (3) that noncoincident kinetics were observed when folding was monitored by different probes.

Schmid (1983) performed a series of experiments to resolve this controversy. He confirmed the existence of I_N on the major slow refolding pathway by showing that it unfolded about 15 times faster than native protein and provided additional data to support the conclusion that the $I_N \rightarrow N$ step was rate-limited by proline isomerization. In addition, he showed that in the course of the slow refolding reaction, the concentration of I_N goes through a maximum and the formation of N displays a lag in the beginning as expected for a sequential reaction. These data confirm that the slow refolding process is sequential and does not involve multiple unfolded states in equilibrium converging to a single native state without the presence of intermediates.

Refolding in Cryosolvents. The experimental evidence presented by Schmid and others strongly suggests that the folding of RNase A is not a simple, two-state process complicated only by the

cis/trans isomeric configurations of the proline residues, but is also defined by the structure and stability of partially folded intermediate states. Exploring this premise, Biringer and Fink (1982, 1988a, b, c) performed a series of experiments with a variety of different structural probes and techniques to monitor the folding of RNase A in methanol-based cryosolvents at subzero temperatures. With the exception of some of the NMR experiments (Biringer and Fink, 1982), most experiments were performed at both pH 6.0 and 3.0 in 35% methanol at -15°C . The low temperatures and methanolic solvent were used to stabilize normally transient intermediates (see *Modified Experiments*) in the folding pathway. The results obtained from these studies (see Table 1) provide a more detailed model for the folding pathway.

NMR studies of RNase A folding (Biringer and Fink, 1982) using the change in peak areas for the C-2 histidine protons (see *Tritium Exchange, NMR and Catalytic Activity*) revealed a native-like intermediate with compact structure that folded to the native state at a rate faster than the proline isomerization event. Refolding of the enzyme as monitored by tyrosine absorbance at 286 nm (ΔA_{286}) was found to be tetraphasic at both pH 3 and pH 6 (Biringer and Fink, 1988c). Refolding as monitored by the change in tyrosine fluorescence was biphasic at both pH values with rates comparable to the two slowest phases observed by ΔA_{286} . Rates and amplitudes were pH independent for the fluorescence probes while rates and amplitudes varied with pH for the absorbance probes. Mono-, di- and trinitrotyrosyl derivatives were synthesized (see *Chemical*

A:

pH 3.0

Signal	$k_I \times 10^2$	$K_{II} \times 10^2$	$k_{III} \times 10^3$	$k_{IV} \times 10^4$
ΔA_{286}	6.2 ± 0.9	$1.3 \pm 0.4(19)$	$1.7 \pm 0.4(19)$	$2.9 \pm 1.0(19)$
Fluorescence	ND	-	$1.3 \pm 0.4(19)$	$3.0 \pm 1.0(7)$
115-NO ₂	2.6 ± 0.6	$0.88 \pm 0.2(37)$	-	-
115, 76-NO ₂	2.3 ± 1.0	$1.4 \pm 0.2(37)$	$0.82 \pm 0.07(9)$	-
76-NO ₂	ND	$1.8 \pm 0.6(40)$	$0.71 \pm 0.06(7)$	-
115,76,73-NO ₂	-	3.0 ± 0.7	$0.75 \pm 0.1(26)$	$1.3 \pm 0.4(18)$
2'-CMP	-	$2.1 \pm 0.5(9)$	-	$3.4 \pm 0.8(13)$
2'3'-CMP	ND	ND	$3.2(11)$	$2.4(15)$
His C2	ND	ND	$2.6(37)$	-
73-NO ₂	ND	-	$1.8 \pm 0.7(41)$	-

pH 6.0

Signal	$k_I \times 10^2$	$K_{II} \times 10^2$	$k_{III} \times 10^3$	$k_{IV} \times 10^4$
ΔA_{286}	5.4 ± 1.2	$1.0 \pm 0.2(-8)$	$3.3 \pm 0.9(9)$	$7.3 \pm 1.0(8)$
Fluorescence	ND	-	$1.7 \pm 0.1(19)$	$3.1 \pm 0.3(5)$
115-NO ₂	8.5 ± 1.0	$1.6 \pm 0.3(26)$	-	-
115, 76-NO ₂	11.0 ± 2.0	$1.6 \pm 0.4(12)$	$2.1 \pm 0.6(8)$	-
76-NO ₂	ND	$1.1 \pm 0.1(15)$	$1.8 \pm 0.1(10)$	-
115,76,73-NO ₂	4.0 ± 2.0	$0.7 \pm 0.06(13)$	$1.5 \pm 0.5(10)$	-
2'-CMP	-	$1.8 \pm 0.3(19)$	$2.5 \pm 0.3(5)$	-
2'3'-CMP	-	ND	-	$3.4(23)$

B:

Signal	$k \times 10^{-3}$	$k \times 10^{-4}$	$k \times 10^{-5}$
RNase	1.7 (19)	3.1(5)	-
115-NH ₂	6.1(57)	1.4(15)	5.3(-23)
115, 76-NH ₂	1.7(14)	3.5(-29)	7.2(61)

TABLE 1: Kinetics of refolding of RNase A and derivatives, -15°C. 35% methanol. Data taken from A: Biringer and Fink (1988a, b, and c), B: Puntambaeker (1992).

Modifications) and characterized by Biringer and Fink (1988a). Kinetics for the refolding of the mononitro-tyrosyl derivative ($C^{\epsilon 115}$ - NO_2 RNase) were biphasic at both pH values while the dinitro- ($C^{\epsilon 115}$, 76 - NO_2 RNase) and the trinitro- ($C^{\epsilon 115}$, 76 , 73 - NO_2 RNase) derivatives were both triphasic. Kinetics for refolding as monitored by nitro-Tyr-76 and nitro-Tyr-73 were obtained by subtraction of the appropriate data sets. The data show that the environment about Tyr-115 becomes native-like faster than that about Tyr-76 and Tyr-73, which in turn fold faster than other regions. This indicates that different regions of the molecule fold at different rates and thus implies a sequential folding pathway in which partially folded intermediates exist. Using the return of catalytic activity (see *Tritium Exchange, NMR and Catalytic Activity*) as a probe for refolding (Biringer and Fink, 1988b), biphasic kinetics were observed at pH 3, whereas only a single phase was found at pH 6. The rate of binding for the competitive inhibitor, 2'-CMP, was biphasic at both pH 3 and 6 with the rate of the faster phase being pH-independent and that of the slower phase was faster at pH 6 than at pH 3. Proline isomerization assays and computer simulations of the data (Biringer and Fink, 1988c) suggest that slow folding occurs through two parallel pathways (minor and major). Thirty percent of the slow-folding molecules follow the minor slow-folding pathway and fifty percent of the refolding protein molecules follow the major slow-refolding pathway. The remaining 20% is attributed to the fast-folding species (U_F) that folds too fast to be observed under their experimental conditions. Combining the results from their

experiments, they proposed the following scheme:

The fastest kinetic transient associated with the major slow-refolding pathway as detected by the nitro-Tyr-115 derivative has a rate of $k = 2.5 \times 10^{-2} \text{ s}^{-1}$ at pH 3 and increases to $k = 1 \times 10^{-1} \text{ s}^{-1}$ at pH 6. The next transient has a rate of $k = 1.3 \times 10^{-2} \text{ s}^{-1}$ at pH 3 and $k = 5.4 \times 10^{-1} \text{ s}^{-1}$ at pH 6. In each case, the rate at pH 6 is four times that at pH 3. The slowest transient is observed by unfolding assays and absorbance at 286 nm and has a rate of $k = 3 \times 10^{-4} \text{ s}^{-1}$ at pH 3 and $k = 7 \times 10^{-4} \text{ s}^{-1}$ at pH 6. The I'_1 intermediate is thought to be a compact species with around 33% native environment in the region of Tyr-115 and Tyr-76. I'_2 is a very native-like intermediate based on the fact that it has full catalytic activity, is capable of binding inhibitor, and is native-like by NMR. I'_2 differs from N by its near UV absorbance (hence, tertiary structure) and it also unfolds much more rapidly than the native state. Conversion of I'_2 to N is a slow process which resembles proline isomerization except that the rate is pH dependent.

In the minor slow-folding pathway, it is assumed that the pH dependence of the individual steps are different, whereas in the major slow-folding pathway, there is a small increase in rate for each step from pH 3 to 6. The fastest transient observed in the

minor slow-folding pathway is detected in the folding about nitro-Tyr-115 and nitro-Tyr-76, inhibitor binding, and tyrosine absorbance of RNase A at 286 nm. The observed rates are: $k = 1.5 \times 10^{-2} \text{ s}^{-1}$ and $k = 6 \times 10^{-2} \text{ s}^{-1}$ at pH 6 and 3, respectively. The next phase includes transients with rates of $k = 1-2 \times 10^{-2} \text{ s}^{-1}$ as determined by inhibitor binding and absorbances of nitro-Tyr-115 and nitro-Tyr-76. The next phase involves transients with rates of $k = 1-3 \times 10^{-3} \text{ s}^{-1}$ and, based on differences in their pH dependencies, may include two different underlying steps. This step was observed by proline isomerization assays, absorbance at 286 nm, tyrosine fluorescence, catalytic activity, and NMR. The slowest phase has a rate of $k = 3 \times 10^{-4} \text{ s}^{-1}$ as detected by fluorescence, inhibitor binding and catalytic activity. The I_3 intermediate is shown to be very native-like by NMR, inhibitor binding and catalytic activity, and it is postulated to differ from the native protein by isomerization state of Pro-93. The I_2 intermediate is native-like in the region of Tyr-115, but the region around Tyr-76 is not native-like and the intermediate has only 75% of the inhibitor binding activity exhibited by the native protein.

As a continuation of these folding studies, Puntambaeker (1992) synthesized, purified and characterized the mono- and diaminotyrosyl derivatives ($C^{\epsilon 115}\text{-NH}_2$ RNase and $C^{\epsilon 115, 76}\text{-NH}_2$ RNase) of RNase A (see *Chemical Modifications*). Refolding of these derivatives in cryosolvents at subzero temperatures yielded triphasic kinetics (see Table 1) at both pH 3 and 6. It is interesting that the rates were an order of magnitude slower than those observed for the corresponding nitro-derivatives. The slowest phase

is thought to be associated with the isomerization of Pro-114 as monitored by the fluorescence of the C^{ε115}-NH₂ RNase.

Refolding of C^{ε73}-NH₂ RNase in Cryosolvent. Currently, data obtained for the change in conditions about Tyr-73 during folding relies upon subtraction of data set for the folding of C^{ε115, 76}-NO₂ RNase from that obtained for C^{ε115, 76, 73}-NO₂ RNase. Manipulations required to obtain this data most probably introduce error and are not as reliable as direct measurement of the environment about Tyr-73 during the folding process.

The goal of the present study was to first synthesize, characterize and purify C^{ε73}-NH₂ RNase and then to examine the folding kinetics under conditions employed by Biringer and Fink. Synthesis was modeled on the work of Wilichek and Gorecki (1969) and Biringer (1985). Several improvements were examined, including the use of the water soluble carbodiimide, EDC, as opposed to dicyclohexylcarbodiimide (DCC). The goals were to improve yields and to eliminate the use of pyridine as a solvent. Characterization of the structure of the amino derivative was made with NMR, isoelectric focusing gel electrophoresis, absorbance and fluorescence spectroscopy. Catalytic activity was examined and compared to native RNase A. The folding kinetics of C^{ε73}-NH₂ RNase were examined at -15 °C in methanolic cryosolvent and compared with results obtained by Biringer and Fink and Puntambaeker.

MATERIALS AND METHODS

2.1 Synthesis of 5'-(4-azophenyl phosphoryl)-uridine

Reagents. Bovine Pancreatic Ribonuclease A, Uridine 5'-monophosphate (UMP) disodium salt, N,N'-Dicyclohexylcarbodiimide (DCC), p-Nitrophenol (PNP) and QAE-Sephadex A-25 (QAE-25) anion exchange resin, Dowex 50X8 cation exchange resin, Sephadex PC-25 (SPC-25), Sephadex G-25 (G-25) gel filtration resin, Palladium on charcoal, sodium nitrite, boric acid, sodium bicarbonate, sodium dithionite, and deuterium oxide were all purchased from Sigma Chemical Company, St. Louis, MO. Affigel-10 affinity resin (Affigel-10) was obtained from Bio-RAD Laboratories, Richmond, CA. Servalyte Precotes isoelectric focusing gels were obtained from Crescent Chemical Company, Hauppauge, NY.

Equipment. Fluorescence measurements were made using a SPEX DM1B Spectrofluorometer and absorbance measurements were made using a Hewlett-Packard 8452A Diode-Array Spectrophotometer. Ultrafiltration was performed in a MFS Ultrafiltration Chamber using Diaflo P-10 ultrafiltration filters. Nuclear Magnetic Resonance (NMR) spectra were obtained on a Bruker MSL 300 NMR Spectrometer. Temperature measurements were made using an Omega Model HH-71T Microcomputer Thermometer equipped with a remote probe.

Preparation and Purification of 5'-(4-nitrophenyl phosphoryl)-uridine (PNPU) by DCC Coupling. The disodium salt of

UMP was converted to the free acid by passing it through a column of Dowex 50X8 (H^+ form). The eluate was frozen and lyophilized and then 0.500 g (1.55 mmol) of the free acid was dissolved in 5 mL of dry pyridine (dried over molecular sieve). The solution was brought to dryness on a rotary evaporator and this process repeated twice more to completely remove the last traces of water from the UMP. A 1.7 g (12.22 mmol) sample of PNP was dissolved in 10 mL of dry pyridine and brought to dryness on a rotary evaporator. As above, the process was performed three times yielding a pale-yellow crystalline material. The dried UMP and PNP were dissolved in 15 mL of dry pyridine and 2.4 g (11.63 mmol) of DCC was added. The reaction vessel was sealed with parafilm and allowed to react, with stirring, for 3-5 days at room temperature.

After the reaction, the mixture was rotovaped to dryness and the residue was slurried in 20 mL of acetone. An equal volume of water was added and the resulting chalky mixture was stirred for 10 minutes. The mixture was filtered through a fritted glass funnel the solid dissolved in a minimum of acetone, and the procedure repeated twice more. The combined aqueous fractions were rotovaped to remove acetone and the pH was adjusted to 3.5 with glacial acetic acid. The solution was extracted with an equal volume of diethyl ether; the ether fractions were discarded. Extractions were repeated until the ether fraction no longer yellowed upon addition of concentrated ammonium hydroxide signifying that most of the PNP was removed. The aqueous fraction was frozen and lyophilized.

After ether extraction, the product still remained contaminated with substantial amounts of PNP, UMP, 1, 3-dicyclohexylurea (DCU) and reaction by-products. Chromatography was required to separate the product from these contaminants. A 100-150 mg sample of the crude product was dissolved in a minimum of 0.01M Triethylamine (TEA) in water with the pH adjusted to 5.0 with acetic acid and then loaded on a 2.5 X 70 cm column of sephadex QAE-25 previously equilibrated with 0.01 M TEA, pH 5.0. The product was eluted with a gradient of 0.01-0.25 M TEA (pH 5.0). The fourth major fraction (see Results and Discussion) was collected and the product concentration was determined by the UV absorbance at 262 nm ($\epsilon=15,000$) in aqueous solution (pH 7.0). Purity was estimated spectroscopically by using the ratio of the absorbance at λ_{max} 262nm ($\epsilon=15,000$) to λ_{infl} 292 ($\epsilon=8200$) at pH 7.0 (Borden and Smith, 1966). Yields ranged from 6 to 8%.

Preparation of and Purification PNPU by EDC Coupling. 1.92 g (10 mmol) of EDC and 1.39 g of PNP (10 mmol) were dissolved in 30 mL of water and stirred for 5 minutes. 0.368 g of 5'UMP (1 mmol) and 10 mL of methanol (to dissolve the PNP) were added to the mixture and the reaction was left overnight at room temperature with constant stirring.

The reaction mixture was adjusted to pH 3.5 with 1.0 M HCl and extracted with equal an volume of diethyl ether; the ether fraction was discarded. Extractions were repeated until the ether fraction no longer yellowed upon the addition of concentrated ammonium hydroxide. The pH was readjusted to 3.5 after each extraction. The

extracted reaction mixture was frozen and lyophilized and a brittle, yellowish, hygroscopic solid was recovered.

As per purification of the DCC reaction mixture, 100-150 mg of the EDC reaction product was resolved using a 2.5 X 70 cm column of sephadex QAE-25. However, the product was eluted with 0.10 M ammonium acetate at pH 5.0. Yields were not calculated.

Preparation of 5'-(4-aminophenyl phosphoryl)-uridine.

Approximately 50 mg of PNPU was dissolved in 20 mL of 80% methanol/water (v/v) and 50 mg of palladium on charcoal (10%) was added. Hydrogen gas was bubbled through the solution for two hours and progress of the reaction was monitored by viewing the UV spectra of aliquots. Conversion of the nitro to amino group results in a reduction of the absorbance of the shoulder at 292 nm in the UV spectrum. The reaction was considered complete when no further change in absorbance at 292 nm occurred. The solution was then filtered through a syringe fitted with a nylon filter (0.45 μ m) to remove the catalyst. The filtrate was rotovaped to remove methanol, and then frozen and lyophilized. The product, 5'-(4-aminophenyl phosphoryl)-uridine (PAPU), was a bright, yellow-orange powder. UV spectra gave λ_{max} at 262 nm at pH 7.0 ($\epsilon=6200$).

Preparation of 5'-(4-diazophenyl phosphoryl)-uridine.

Approximately 40 mg (0.12 mmol) of PAPU was dissolved in 1.0 mL of 1 M HCl and chilled in an ice bath. Three aliquots of 0.1 M sodium nitrite (0.333 mL each) were added very slowly. The reaction was allowed to proceed for 15 minutes and then quenched by the addition of 1.0 mL of chilled borate buffer (pH 8.5). The solution was allowed to warm to room temperature and 1 M sodium hydroxide was used to

adjust the pH to 8.5 where the solution turned a bright orange color. The azo compound was not isolated and the solution was used immediately for the azotization of RNase A.

2.2 Synthesis of C^ε73-NH₂ RNase

Purification of RNase A. A SPC-25 column was equilibrated with 0.15 M phosphate buffer, pH 6.5 and 223.4 mg of RNase A was dissolved in 3 mL of water and loaded on the column. The column was eluted with 0.15 M phosphate buffer and the first peak (aggregated protein) was discarded. The second peak contained the pure monomeric RNase A and these fractions were pooled and dialyzed for 24 hours against 20 L of deionized water. Dialysis was repeated twice more. The dialyzed product was frozen and lyophilized, yielding 159 mg of purified RNase A.

Preparation of Azotyrosyl RNase Derivative. A 100 mg sample of purified RNase A was dissolved in 3.0 mL of 0.1 M borate buffer (pH 8.5). The buffered RNase A solution was added to 3.0 mL of a freshly prepared solution (from *Preparation of 5'-(4-diazophenyl phosphoryl)-uridine*) of 0.12 mM 5'-(4-azophenyl phosphoryl)-uridine and allowed to react for 3 hours with stirring. The solution was loaded on a Sephadex G-25 column (2.5 X 30 cm) and eluted with 0.1 M ammonium hydroxide. Two peaks were observed: the first had a λ_{max} of 278 nm and represents the azotized protein and the second had a λ_{max} of 262 nm and contained the unreacted azophenyl phosphoryl uridine. The protein fractions were pooled, frozen and

lyophilized.

Preparation of C^{ε73-NH₂} RNase. The azo protein was dissolved in 5 mL of 0.1 M sodium bicarbonate. Two milliliters of freshly prepared 0.1 M sodium dithionite solution were added and the reaction was allowed to proceed for 10 minutes with constant stirring. The C^{ε73-NH₂} RNase was separated by passing the solution through a G-25 column (2.5 X 30 cm) with deionized water as the eluant. The protein fraction (approximately 40 mL) was collected and concentrated to approximately 10 mL in an ultrafiltration chamber fitted with a P-10 filter and pressurized with 40 psig of nitrogen. The sample was diluted and concentrated several times to insure removal of impurities. The concentrate was frozen and lyophilized. At this point, PAPU is still bound in the active site of the C^{ε73-NH₂} RNase is removed with an RNase-specific affinity column.

Preparation of an RNase-specific Affinity Column.

Approximately 3 mL of Affigel-10 affinity resin were transferred to a fritted glass funnel and washed with deionized water. The Affigel-10 resin had a theoretical binding capacity of 10 μmol of PAPU per mL of gel. A sample containing 0.092 mmol of PAPU in water (determined spectrophotometrically) was diluted to 5.0 mL with deionized water. This was added to the Affigel-10 resin and allowed to react over night. The excess PAPU was used to insure maximum binding to the resin. The lengthy reaction time insured that hydrolysis of any unreacted sites on the resin had occurred. The mixture was poured in a column (1 X 10 cm) and washed with

deionized water until free of contaminants as determined by UV absorbance of the eluant. Another portion of resin was reacted with PAPU and added to the column so that the final volume of resin was approximately 3 mL.

The column capacity was determined by measuring the amount of purified RNase A that would bind to the column. Fifty mg of RNase A was dissolved in 10 mL of distilled water and loaded on the column in 5 mg (1 mL) increments with distilled water washes between increments. Bound RNase A was eluted from the column with 0.20 M acetic acid and RNase A concentration determined spectrophotometrically ($\epsilon = 9,800$ at 278 nm, pH 7.0). Column capacity was determined to be 37.8 mg RNase A (see Results and Discussion).

Purification of C^{E73}-NH₂ RNase. Approximately 10 mg of C^{E73}-NH₂ RNase was dissolved in 5.0 mL of distilled water and passed through the affinity column. The column was washed with 20 mL of distilled water which removed PAPU displaced from the bound protein and any protein that did not bind to the column. Bound protein was then eluted from the column with 0.2 M acetic acid. The remaining C^{E73}-NH₂ RNase was purified (in 10 mg lots) with the affinity column as noted above. Fractions containing the bound protein from all the separations were pooled, dialyzed against 20 L distilled water three times, frozen and lyophilized.

2.3 Characterization C^{E73}-NH₂ RNase

Spectroscopic Properties. A molar extinction was determined

using a 0.929 mg/mL solution of purified C^{ε73}-NH₂ RNase in 0.033 M acetate buffer, pH 7.0. Sample gave a λ_{max} of 278 nm with a calculated molar extinction of 11,842.

Fluorescence spectra were obtained using 0.517 mg/ mL C^{ε73}-NH₂ RNase in 0.033 M Acetate buffer, pH 6.0. A broad peak with a maximum between 340 to 350 nm was observed with excitation at 288 nm and an emission maximum at 310 nm was observed with excitation at 278 nm.

NMR. Amide protons were exchanged by reaction with deuterium oxide. A 12 mg sample of C^{ε73}-NH₂ RNase was dissolved in 2.00 mL of deuterium oxide and the pH adjusted to 3.0 with deuterium chloride (DCI). The measurement was made with a standard combination electrode and no adjustment was made for the isotope effect. Samples were incubated at 60°C for 1 hour, allowed to cool to room temperature, and then frozen and lyophilized. Samples for nmr experiments were dissolved in 1.000 mL of 0.10 M KCl in deuterium oxide (100%) with pH adjusted to either 3.0 or 7.0 with DCI or NaOD (sodium deuterioxide). Fifty mg of 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS, sodium salt) was added to each sample for a reference peak.

Isoelectric Focussing Gel Electrophoresis. Solutions of 5 mg/mL of C^{ε73}-NH₂ RNase and purified RNase A in distilled water were prepared. Sample volumes of 2.5, 5.0 and 7.5 μ L of both solutions were applied to a isoelectric focussing gel, 1.25 X 1.25 cm, (Servalyte PrecoteTM, pH 3-10) along with pH markers. The gel was developed on a BIO-PHORESISTM horizontal electrophoresis cell at a

constant 4 W power with cooling (4°C). The gel was fixed with trichloroacetic acid and then stained with Coomassie Blue to visualize the proteins.

Catalytic Activity. Catalytic activity was assessed using the method of Crook *et al.* (1959). Activity for the C^{E73}-NH₂ RNase was calculated relative to activity of purified RNase A as determined by the assay.

2.4 Refolding of C^{E73}-NH₂ RNase

Cryostatic Buffers and stock solution. Refolding of C^{E73}-NH₂ RNase was determined by measuring the fluorescence change versus time at pH 6.0 and pH 3.0 at -15° C. This required the use of buffers containing methanol to prevent freezing. Cryostatic acetate buffer (0.033 M) was prepared with 35% methanol/distilled water and the pH adjusted to 6.0. Formate buffer (0.033M) was prepared in a similar fashion and the final pH was adjusted to 3.0. Solutions containing 0.75 mM of C^{E73}-NH₂ RNase were made up in both cryostatic acetate and formate buffers to serve as stock protein solutions. 6 M Guanidinium chloride (GdmCl) solutions were made up in cryostatic acetate and formate buffers for use in denaturing protein.

Specialized Equipment. As fluorescence measurements were made at subzero temperatures, a custom-built cryostatted cell holder with a large insulated cooling block was used. This cell holder was ported so that dry nitrogen gas could be directed on the

surface of the sample cells to prevent condensation. Cooling lines for the cell holder were insulated and connected to a large capacity cooling bath and the temperature was thermostatically controlled by a remote temperature controller. A 1 cm fluorescence cell with 1.000 mL of cryosolvent was placed in the cell holder and the temperature was adjusted to give -15°C in the cell as measured with a microcomputer thermometer. The sample cell was fitted with a teflon top that had two small holes: one for the temperature probe and one to accept the glass syringe for injections of protein and a battery-powered cell stirrer for initial mixing of the injected protein.

The Spex DM1-B control module for the fluorometer did not offer the flexibility needed for manipulating and printing out the refolding fluorescence data. As a result, an RS-232 output from the fluorometer was used to transfer the data to a 386 IBM clone. Data was analyzed and graphics produced by an ExcelTM spreadsheet.

Unfolding and Refolding Procedures. Regardless of the buffer used, all refolding experiments were performed in the same manner. 50 μL of the protein stock solution were drawn into a glass micro-syringe and placed in a 70°C water bath for 10 minutes. These conditions are known to completely unfold the protein (Biringer and Fink, 1982).

The unfolded sample was injected into the sample cell containing 1.000 mL of cryostatic buffer at -15°C and fluorimetric measurements were initiated immediately. The cell was stirred for 20 seconds using a motorized cell stirrer. Data was acquired using

measurements to be made (using set excitation and emissions wavelengths) at selected time intervals. Temperature was monitored by the microcomputer thermometer and recorded on a strip chart recorder during the entire course of the refolding reaction.

Evaluation of Kinetic Data. Semilog plots of the data (log of the change in fluorescence versus time) gave a straight line for the data showing that the observed folding followed simple first order kinetics. Linear regression analysis was used to evaluate the fit of the line to the data. In addition, the raw data was fit to a single exponential by NFIT™ using the function:

$$F_t = -A_1 e^{-tk_1} + A_{inf}$$

where t is equal to time, F_t is the observed fluorescence at time t , A_1 is the amplitude of the change, k_1 is the rate constant, and A_{inf} is the amplitude of the folded protein.

RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of C^{E73}-NH₂ RNase

Method of Wilchek and Gorecki, and Biringer. Using diazonium salts as coupling agents, Wilchek and Gorecki (1969, 1978) developed a novel procedure for the amination of Tyr-73 of RNase A in the epsilon position. Their method is based on modifying the nucleotide uridine-2'(3'), 5'-diphosphate, a strong competitive inhibitor for RNase A ($K_i = 10\mu\text{M}$). A three-step synthesis is used to modify this nucleotide so that it can be used to aminate Tyr-73. First, p-nitrophenol (PNP) is coupled to the nucleotide with dicyclohexycarbodiimide (DCC), forming 5'-(4-nitrophenyl-phosphoryl)-uridine-2'(3')-diphosphate. Then, the nitro group is reduced with hydrogen over palladium on charcoal to give 5'-(4-aminophenyl phosphoryl)-uridine-2'(3')-diphosphate. Finally, the diazonium salt is obtained by reaction of the amino derivative with sodium nitrite in HCl.

When this diazonium salt of the modified inhibitor is introduced into the active site of RNase A, the phenolic group acts as a "spacer arm" and positions the diazonium group to selectively couple with Tyr-73 in the epsilon position. Subsequent reduction of the diazo bond produces C^{E73}-NH₂ RNase. Removal of the inhibitor

from the active site of the modified RNase A is accomplished using an affinity column with 5-(4-aminophenyl phosphoryl)-uridine-2(3)-phosphate (obtained from the reduction of the nitrophenyl nucleotide) as a ligand. This nucleotide shows a strong affinity for RNase A ($K_i = 70\mu\text{M}$). As the RNase-Inhibitor complex passes through the column, the bound inhibitor nucleotide is displaced from the active site by the bound ligand.

Characterization of C^{18}NH_2 RNase produced by this method showed that it retained catalytic activity while amino acid analysis of the compound confirmed the stoichiometry and specificity of the synthesis.

Biringer (1985) modified this procedure by substituting the nucleotide, uridine-5'-monophosphate (5'-UMP), for 5'-phosphoryl-uridine-2'(3')-cyclic phosphate in the synthesis (see Figure 2: Scheme one, two and three). Although the affinity of this compound for the active site is slightly less than that of the cyclic phosphate, it is more than adequate to insure the desired specificity.

Synthesis and Purification of PNPU: DCC Coupling. PNPU was synthesized using the method of Biringer (1985). The DCC coupling of PNP and UMP (see Figure 2. Scheme One) in pyridine was performed under anhydrous conditions. Water, if present, would react with the DCC (Khorana, 1953) to produce 1,3-dicyclohexyl urea (DCU), consuming the DCC and lowering the yields. As a result, UMP and PNP were dissolved in dry pyridine and rotovaped to dryness several times to remove any moisture. In some cases, it was observed that PNP would produce an oil instead of yellow crystals when dried in

FIGURE 2. Scheme 1: DCC coupling of 5'-UMP and PNP

FIGURE 2. Scheme 2: Reduction of PNPu to PAPu

5'-(4-diazophenyl phosphoryl)-uridine

FIGURE 2. Scheme 3. Azotization of PAPU

pyridine. When this oil was used in the coupling reaction, little or no PNP was formed. After initial removal of DCU from the reaction mixture using acetone and water, the aqueous phase was extracted with ether to remove unreacted PNP.

PNPU, synthesized by Borden and Smith (1966) using a DCC coupling reaction, yielded a product (see Figure 4) with UV absorptions at $\lambda_{\max}$ 261 nm ($\epsilon = 15,000$), $\lambda_{\inf}$ 292 nm ($\epsilon = 8,200$) and $\lambda_{\min}$ 233 ($\epsilon = 4,500$) at pH 7.0 and $\lambda_{\max}$ 261 nm ($\epsilon = 12,500$), $\lambda_{\inf}$ 292 nm ($\epsilon = 8,200$), and $\lambda_{\min}$ 239 nm ($\epsilon = 7,300$) at pH 12.0. Comparison of this data with the spectrum of UMP, which gives UV absorptions at $\lambda_{\max}$ 262 nm ($\epsilon = 10,000$), $\lambda_{\min}$ 232 at pH 7.0, shows that the shoulder at 292 nm results from an overlap of absorbance of the nitrophenol group ($\lambda_{\max}$ 318 nm for protonated species). These data were used to identify and assess the purity of the PNPU.

It was found that the ether extractions did not adequately purify the product and it remained contaminated with PNP and UMP. Various attempts at separation by chromatography using silica gel with a variety of systems met with little success. QAE-25 was found to separate the components of the reaction mixture using several different solvent systems (ammonium chloride, ammonium acetate, etc.). Although volatile, these buffers contaminated the lyophilized product and were difficult to remove. It was discovered that a gradient of 0.01 to 0.25 M triethylamine adjusted to pH 5.0 with acetic acid offered excellent separation of the reaction mixtures and had the added advantage of being completely volatilized during the lyophilization process. The chromatogram of a

typical separation is found in Figure 3. The major product (major peak four) was confirmed to be PNPU by UV spectra (see Figure 4). Peaks two and three were unreacted PNP and UMP, respectively. Yields were low, varying from 6 to 8% as compared to the yields for PNPU reported by Borden and Smith (25%) and the yields reported for the 5'-(4-nitrophenyl phosphoryl)-uridine-2'(3')-phosphate (85%) by Wilchek and Gorecki (1969). This low yield was probably due to the contamination of the reaction mixture with water and the production of N-phosphorylureas. Multiple syntheses were performed and the products recovered were combined to provide a quantity of PNPU sufficient for further experiments.

Synthesis of and Purification of PNPU: EDC Coupling. The water-soluble coupling agent, 1-(3-Dimethylethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Keeton, Krutzsch and Lovenberg, 1981), was selected as a substitute for DCC in the synthesis of PNPU in an attempt to improve yields, eliminate the use of pyridine and reduce reaction times. The stability of EDC in water (Gilles, Hudson and Borders, 1990) has been reported to be a function of pH and the stability drops dramatically below pH 6.0. Reaction conditions were designed with this in mind.

The first experiments were performed by dissolving the reactants in distilled water. Sodium hydroxide was added to solubilize the PNP. The reaction resulted in the formation of a thick viscous material (probably due to the polymerization of UMP). Later experiments used a mixture of methanol and water to improve the solubility of PNP without raising the pH. This approach

FIGURE 3: Chromatogram of DCC reaction mixture on 2.5 X 70 cm QAE-25 column eluted with 0.01 to 0.25 M triethylamine at pH 5.0. Tick marks represent individual fractions (120 drops/tube or approximately 6 mL/tube).

FIGURE 4: 35 μ M PNPU in 0.033M acetate buffer at pH 7.0

minimized the formation of the viscous material. Attempts to purify this reaction mixture by ether extraction left a mixture of products and reactants. The mixture was successfully resolved using a QAE Sephadex-25 (QAE-25) column developed with 0.1 M ammonium acetate (pH 5.0). Figure 5 shows that the reaction mixture was separated into six major peaks. Spectra of these peaks were used to identify peak 3 as PNP, peak 4 as UMP, and peak 5 as PNPU. The major product, peak 6 from figure 5, had a λ_{max} at 266 nm and an inflection at 292 nm. This compound contained both the PNP and perhaps uridine groups but it differed from PNPU in the absorbance maximum at 266 instead of 262 nm and it was also more polar, since it was retained longer on the column. The yield of PNPU (peak 5) was very small in relation to the unreacted UMP (peak 4) and the unknown major product (peak 6) so this approach was abandoned in favor of using the DCC coupling method.

Preparation of the 5'-(4-aminophenyl phosphoryl)-uridine (PAPU). Reduction of PNPU with Pd on charcoal yielded PAPU with minimal side products although slight traces of UMP and PNP (from hydrolysis of PNPU) were detected. A typical UV spectrum of PAPU (see Figure 6) shows that the shoulder occurring at 292 nm is greatly reduced in relationship to the λ_{max} at 262 nm as shown by the corresponding nitro derivative (see Figure 4).

Modification of RNase A Using 5'-(4-azophenyl phosphoryl)-uridine. PAPU was converted to 5'-(4-azophenyl phosphoryl)-

FIGURE 5: Chromatogram of EDC reaction mixture on 2.5 X 70 cm QAE-25 column eluted with 0.10 M ammonium acetate at pH 5.0. Tick marks represent individual fractions (120 drops/tube or approximately 6 mL/tube).

FIGURE 6: UV spectrum of 35 μM PAPU in 0.033 M acetate buffer at pH 7.0.

uridine using sodium nitrite and HCl and the reaction mixture was used immediately for the azotization of RNase A. The results of purification of the modified protein using the G-25 column are shown in Figure 7. The first peak contained diazotized protein which was dialyzed then frozen and lyophilized. This diazo derivative is orange in color (See Figure 8) and has a UV spectra with a λ_{max} at 262nm and at 342 nm which was consistent with the results of Wilcheck and Gorecki (1978).

Preparation and purification of C^ε73-NH₂ RNase . The reaction of the azophenyl phosphoryl uridine with RNase A produces a product with the modified uridine in the active site of the protein that is covalently coupled to the epsilon carbon of Tyr-73 of the RNase A via a diazo bridge (azo-RNase). In order to prepare and purify C^ε73-NH₂ RNase, it was first necessary to reduce the diazo linkage to amino groups and then to remove the resulting PAPU from the active site.

Reduction was accomplished using sodium dithionite and the reaction mixture was quenched and resolved on a G-25 column using water as an eluant. Figure 9 shows the results of this separation: the first peak was C^ε73-NH₂ RNase and the second peak was the dithionite (as identified by UV spectra) and buffer (sodium bicarbonate). Ultrafiltration, dialysis and lyophilization of the protein fraction produced the C^ε73-NH₂ RNase with PAPU still bound, albeit not covalently, in the active site.

The protein was purified by passing it through an affinity

FIGURE 7: Chromatogram of diazotization reaction mixture on 2.5 X 16 cm G-25 column eluted with 0.10 M ammonia. Tick marks represent individual fractions (120 drops/tube or approximately 6 mL/tube).

FIGURE 8: UV spectrum of 35μM Azo-RNase in 0.033 M acetate buffer at pH 7.0.

FIGURE 9: Chromatogram of reduced Azo-RNase reaction mixture on 2.5 X 17 cm G-25 column eluted with deionized water. Tick marks represent individual fractions (120 drops/tube or approximately 6mls/tube).

column where the bound PAPU was displaced by the PAPU ligand of the column. The protein was then eluted with 0.2 M acetic acid. Figure 10 shows the result of this separation. The first peak was the PAPU and the second peak (eluted by the acetic acid) was the protein fraction as determined by UV spectra. After dialysis and lyophilization, the UV spectra of the purified protein (see Figure 11) was consistent with the results of Gorecki and Wilchek (1978) with λ_{max} at 278 nm ($\epsilon = 11,842$) and a weak shoulder at around 288 nm.

3.2 Characterization of C^ε73-NH₂ RNase

NMR Spectra. NMR spectra were obtained for amino tyrosine as well as histidine, phenylalanine and tyrosine at pH 7.0. Figure 12 (upper spectrum) shows that the NMR spectrum of aminotyrosine consists of two doublets centered around 7.05 and 7.2 ppm and a overlapping singlet at 7.25 ppm. Figure 12 (lower spectra) shows overlapping spectra of tyrosine, phenylalanine and histidine under the same conditions. It can be seen that the aminotyrosine doublets are shifted in relation to tyrosine as a consequence of the amino group.

Proton NMR spectra of proteins were enhanced by the convolution difference enhancement method which uses Lorentz-Gauss multiplication (Ferrige and Lindon, 1978). This method involves multiplication of the FID by the function: $e^{(-at-be^2)}$.

Figure 13 shows the NMR spectrum of RNase A at pH 3.0

FIGURE 10: Chromatogram of C^{E73-NH_2} RNase on 0.5 X 5.0 cm Affigel-10 column with PAPU ligand. Compounds eluted with deionized water 0.2 M acetic acid (at tube 80. Tick marks represent individual fractions (120 drops/tube or approximately 6mls/tube).

FIGURE 11: UV spectrum of 35 μ M C ^{ϵ} 73-NH₂ RNase in 0.033 M acetate at pH 7.13.

FIGURE 12: Upper: NMR spectrum of aminotyrosine. Lower: NMR spectra of tyrosine (Tyr), histidine (His) and phenylalanine (Phe) in D_2O at pH* 7.0 (measured directly without correction for isotope effect).

FIGURE 13: Upper: NMR spectrum of RNase. Lower: NMR spectrum of C^ε73-NH₂ RNase. Both in D₂O at pH* 3.0 (measured directly without correction for the isotope effect).

(lower spectrum) and the spectrum of C^{ε73-NH₂} RNase (upper spectrum) under the same conditions. It is clear from comparison of the two spectra that the resonances for Tyr-115, Tyr-76 and Tyr-25 are the same for both RNase A and C^{ε73-NH₂} RNase. This confirms that these tyrosine residues were not aminated or the resonances would be shifted. Resonance A (Figure 13, upper spectrum) is unique to C^{ε73-NH₂} RNase and may be associated with the 73-aminotyrosyl group. Additional experiments will be necessary for confirmation.

Isoelectric Focusing. Figure 14 shows the isoelectric focusing gel, pH 3.0 to 10.0, for C^{ε73-NH₂} RNase and RNase A. The results show that the derivatized protein has approximately the same pI as RNase A (9.6) and that the protein is homogeneous.

Catalytic Activity. Catalytic activity was determined using a modification of the method of Crook *et al.* (1959). Activity was determined by comparing the rates ($V_{\max}$) of RNase A versus the derivatized protein using 2',3'-CMP under saturating conditions. The relative catalytic activity for the derivative was found to be 72% which is consistent with results obtained by Biringer and Fink (1988a). They obtained 98% catalytic activity with C^{ε115,76-NO₂} RNase and 74% activity with C^{ε115,76,73-NO₂} RNase. The activity loss upon nitrating Tyr-73 is due to the fact that it is located in a cleft in the protein near the active site where it can interfere with catalysis.

Oxidation of C^{ε73-NH₂} RNase. In earlier experiments (Puntambaeker, 1992), aminotyrosyl RNase derivatives of RNase A (C^{ε115-NH₂} and C^{ε115,76-NH₂} RNase) were observed to oxidize the

FIGURE 14: Isoelectric focusing gel (pH 3.0-10.0)

Lanes A and H: Molecular weight markers (50 μ g)

Lanes B, C, F and G: RNase A (35, 50 and 35 μ g, respectively)

Lanes D and E: C⁸⁷³-NH₂ RNase (35 and 50 μ g, respectively)

corresponding nitrotyrosyl derivatives under mild acidic conditions. The nitro derivatives exhibit very different spectra with an additional peak (λ_{max} at 400-500 nm) and do not fluoresce. As a result, kinetic studies at pH 3 based on the fluorescence of aminotyrosines during protein folding were adversely affected. Experiments were performed to determine if C^ε73-NH₂ RNase was subject to oxidation under the conditions of refolding.

A sample of C^ε73-NH₂ RNase was diluted with water, the pH was adjusted to 3.0 using HCl, and oxygen gas was bubbled through the solution for four hours at a temperature of 30°C. In a parallel experiment, A sample of C^ε73-NH₂ RNase was oxygenated for three and one half hours at a pH of 8.57. Aliquots were removed at various times from these mixtures and their UV spectra examined. No significant change in absorbance in the 400 to 500 nm region occurred during the experiments and thus no appreciable oxidation took place.

Since aminotyrosine-73 is believed to be somewhat "buried" in a hydrophobic pocket, it is conceivable that the aminotyrosyl residue in the native protein is protected from oxidation. However, this aminotyrosyl residue might still be susceptible to oxidation in the unfolded state. Consequently, a sample of C^ε73-NH₂ RNase was added to 8 M urea at pH 3.0 and allowed to unfold for one hour and fifteen minutes. Both absorbance scans and fluorescence scans (excitation at 288 nm) were performed initially, and at 45 and 75 minutes. No new absorbance bands were observed in the UV spectra and no change was observed in the fluorescence spectra. This indicates that amino

Tyr-73 is not susceptible to oxidation even when fully solvent exposed.

During the course of a refolding experiment, the protein is exposed to UV light for several hours. In order to explore the possibility that UV light might catalyze the oxidation event, a sample of C^ε73-NH₂ RNase was dissolved in oxygenated 8 M urea and exposed to UV light in the fluorometer at 290 nm for an hour. Fluorescence scans initially and at the 1 hour interval showed virtually no change in value during this experiment.

These attempts to oxidize C^ε73-NH₂ RNase to the corresponding nitro derivative were all unsuccessful implying that it would be stable under the conditions of the refolding experiments.

3.3 Folding of C^ε73-NH₂ RNase

Experimental Controls and Conditions. It is necessary to insure that fluorescence changes observed during the course of the refolding experiments truly reflect the folding events and are not influenced by external factors such as temperature change and instrumental drift or noise. Maintaining a constant temperature during the course of the experiments was especially important, as fluorescence of the aminotyrosyl residue decreases with increasing temperature.

For each folding experiment, injection of the sample was followed by 20 seconds of continuous stirring. An addition 60 to 80

seconds were required for fluorescence signal to stabilize.

Consequently, measurements made during the initial 100 seconds of the experiment were not used to evaluate the kinetics of folding.

Injection of the hot sample (70 °C) raised the temperature of the folding buffer in the sample cell. The magnitude and duration of this temperature change was monitored to insure that fluorescence measurements faithfully represented folding events and not temperature transients. It was observed that the temperature initially rose to -12.5 °C and returned to -13.8 °C within one minute. Return to -15.0 °C required about three minutes. The temperature after 100 seconds was about -14.5 °C (when the first data was collected). In control experiments, it was observed that the fluorescence of the native and denatured protein (in 6.0 M guanidinium chloride) did not change when the temperature was raised from -15.0 °C to -14.5 °C. As a precaution, temperature was monitored and recorded during all folding experiments to insure that fluorescence changes observed were not temperature related.

The fluorometer baseline was not completely stable over long periods of time. However, the changes in fluorescence attributed to the amplitude of this drift were insignificant over the time period of the refolding experiments. In addition, electronic noise (often in the form of spikes) was removed when present, prior to data analysis.

Fluorescence of C^ε73-NH₂ RNase. The objective of synthesizing the amino derivative of tyrosine-73 was to produce a probe with a fluorescence emission maximum far enough removed

tyrosine 73 could be distinguished from those tyrosines not modified. The fluorescence spectra of C^ε73-NH₂ RNase at pH 6.0 and pH 3.0 under native conditions are shown in Figure 15. The corresponding spectra under denaturing conditions are given in Figure 16. The emission spectra at both pH values exhibit a broad band from around 320 nm to 500 nm with a λ_{max} at 420 nm and a small peak at 340 nm when excited at 300 nm. Excitation spectra of C^ε73-NH₂ RNase at pH 6.0 and pH 3.0 with emission at 420 nm are shown in Figure 17. At pH 6.0, the excitation λ_{max} is between 340 nm to 370 nm and at pH 3.0, the λ_{max} is around 300 nm. Due to the overlap of the excitation and emission spectra in the region around 340 nm and the prominence of the λ_{max} at 420 nm, 420 nm was chosen as the wavelength for monitoring the folding events. This wavelength is far removed from the tyrosine emission $\lambda_{\text{max}} = 305$ nm, and thus should specifically monitor changes in the 73-aminotyrosine region. However, excitation wavelengths of 300 nm or 340 nm could be used to achieve the emission at 420 nm. Excitation denatured C^ε73-NH₂ RNase at 342 nm (pH 6.0) produced the spectra given in Figure 18 which shows that the prominent peak at 420 nm is still present.

In order to determine which excitation wavelength was more suitable and perhaps more revealing, folding kinetics were measured at 420 nm with excitation at both 300 nm and 342 nm for all solvent conditions. Examination of the folding kinetics revealed that both excitation wavelengths gave single exponential kinetics with identical rate constants and similar amplitudes.

FIGURE 15: Fluorescence emission spectra of C ϵ 73-NH₂ RNase (excitation at 300 nm) at pH 3.0 (upper) and pH 6.0 (lower) in 35% methanol at -15°C.

FIGURE 16: Fluorescence emission spectra of C ϵ 73-NH₂ RNase (excitation at 300 nm) at pH 6.0 (upper) and pH 3.0 (lower), both in 35% methanol, 6M guanidinium chloride at -15°C.

FIGURE 17: Fluorescence excitation spectra of C⁸⁷³-NH₂ RNase (emission at 420 nm) at pH 6.0 (upper) and pH 3.0 (lower), both in 35% methanol at -15°C.

FIGURE 18: Fluorescence emission spectrum of C ϵ 73-NH₂ RNase (excitation at 342 nm) at pH 3.0 in 35% methanol, 6M guanidinium chloride.

It is interesting that the excitation spectrum of the protein shifts from a maximum around 340 nm to 370 nm at pH 6.0 to a maximum of 300 nm at pH 3.0 while the prominent emission maximum remains at 420 nm. Aminotyrosine was used as a model compound to investigate this phenomenon. Absorption and fluorescence spectra were obtained for aminotyrosine in water at various pH values between 6.0 and 3.0. Figure 19 shows the emission spectrum of amino tyrosine at pH 6.0 with excitation at the absorption λ_{max} (290 nm) and Figure 20 shows the emission spectrum at pH 3.0 with excitation at the corresponding absorption λ_{max} (276 nm). At pH 3.0, the emission spectrum is split into two peaks with absorption maxima at 340 nm and 310 nm. Excitation spectra of aminotyrosine at both pH 3.0 and 6.0 are given in Figure 21. A shift of approximately 25 nm is observed between the excitation spectra at the two pH values. However, this shift can be explained by protonation of the carboxylic acid group of the aminotyrosine which has a pK value of around 2.5 to 3.0. Hence, this compound is probably not a good model; future experiments will use aminotyrosine ethyl ester in methanolic solvents.

Several aminotyrosyl derivatives of RNase A, C^{ε115}-NH₂ RNase and C^{ε115,76}-NH₂ RNase were prepared by Puntambaeker. The fluorescence spectrum observed for C^{ε115}-NH₂ RNase and C^{ε115, 76}-NH₂ RNase (35mM in 35% methanol/DI water, pH 6.0 in 0.033 M acetate buffer) are shown in Figure 22. The C^{ε115}-NH₂ RNase gave a fluorescence maximum around 420 nm when excited at 288 nm. Thus, the spectral characteristics of the C^{ε73}-NH₂ RNase are similar

FIGURE 19: Emission spectrum aminotyrosine at pH 6.0 (excitation at 290 nm).

FIGURE 20: Emission spectrum of aminotyrosine at pH 3.0 (excitation at 276 nm).

FIGURE 21: Excitation spectrum of aminotyrosine (emission at 350 nm) at pH 6.0 and excitation spectrum of aminotyrosine (emission at 342 nm) at pH 3.0.

FIGURE 22: Curve A: Emission spectrum (1/10 scale) of RNase (excitation at 278 nm). Curve B: Emission spectrum of C^ε115, 76-NH₂ RNase (excitation at 288 nm). Curve C: Emission spectrum of C^ε115-NH₂ RNase (excitation at 288 nm). All spectra taken at pH 6.0 in 35% methanol.

to those of the C^ε73-NH₂ RNase and are also consistent with results reported by Seagle and Cowgill (1976).

Folding Data for C^ε73-NH₂ RNase. Folding experiments performed with C^ε73-NH₂ RNase yielded one measurable kinetic phase at both pH values. Figure 23 shows a typical plot of the raw data for pH 3.0. Figure 24 shows the semilog plot of this data with the regression line for the analysis superimposed over the graph. Data for other experiments at pH 6.0 gave comparable results. Figure 25 shows the plot of the raw data and Figure 26 shows the corresponding semilog plot.

Table 2 gives the results of kinetic evaluations of the data using graphical methods and the NFit™ program. The rate constants for both pH 3.0 and pH 6.0 experiments are essentially identical as are the amplitudes.

Interpretation of Kinetic Data. Table 1 shows the rate constants and amplitudes for folding data obtained by others (Biringer and Fink, 1982, 1988a, b, c, and Puntambaeker, 1992) at -15 °C in 35% methanol at both pH 3.0 and 6.0. The rate constant for C^ε73-NO₂ RNase, obtained by subtracting data sets for the C^ε115,76-NO₂ RNase from those for the C^ε115,76,73-NO₂ RNase, agree closely with values obtained by refolding experiments using C^ε73-NH₂ RNase. The third kinetic phase for the fluorescence-monitored refolding of RNase A (observed at both pH 3.0 and 6.0) has a similar rate and amplitude and is also independent of pH. Results obtained by Puntambaeker for refolding of C^ε115, 76-NH₂ RNase give a rate constant of $1.7 \times 10^{-3} \text{ s}^{-1}$ at pH 6.0 with an amplitude of 14% for

FIGURE 23: Refolding of 35 μM C ϵ 73-NH $_2$ RNase, pH 3.0 in 35% methanol at -15°C.

FIGURE 24: Semilog representation of the refolding for C ϵ 73-NH₂ RNase given in FIGURE 23 (pH 3.0, 35% methanol, -15°C).

FIGURE 25; Refolding of 35 μM C ϵ 73-NH $_2$ RNase, pH 6.0, 35% methanol, -15°C.

FIGURE 26: Semilog representation of the refolding for C^{E73}-NH₂ RNase given in FIGURE 25 (pH 6.0, 35% methanol, -15°C).

pH 3.0

Rate Constant (sec ⁻¹)	Std. Dev.	Amplitude	Rate Constant (sec ⁻¹) N-FIT	SD _{N-FIT}
0.001684	0.000146	11.7%	0.001320	0.00013

pH 6.0

Rate Constant (sec ⁻¹)	Std. Dev.	Amplitude	Rate Constant (sec ⁻¹) N-FIT	SD _{N-FIT}
0.001742	0.00033	12.3%	0.001320	0.00023

TABLE 2: Kinetic data for refolding of C^ε73-NH₂ Ribonuclease A, pH 3.0 and 6.0, 35% Methanol, -15°C.

that the fluorescence of C^ε73-NH₂ RNase monitors the event in the folding pathway that is characterized by k_{III} obtained for fluorescence monitored folding of RNase A.

Recalling the scheme proposed by Biringer and Fink (see Introduction) for the folding pathway of RNase A at subzero temperatures in cryosolvents,

k_{III} is the rate for conversion of I_2 to I_3 on the minor slow folding pathway. According to Biringer and Fink (1988c), this step may actually involve two kinetic processes: one in which the rate and amplitudes are dependent upon pH and one for which rate and amplitudes are independent of pH. The fluorescence of C^ε73-NH₂ RNase monitors the event which is pH independent. At present, the nature of this event is unknown. In addition, Tyr-73 is apparently not sensitive to the isomerization of Pro-114 (slowest phase, Table 2) or Pro-93 (k_{IV} Table 1) since no phase with corresponding rates was observed in these experiments.

Conclusion. C^ε73-NH₂ RNase was prepared using a modification of the synthetic methods of Gorecki and Wilchek (1978) and Biringer (1985). This involved the synthesis of p-nitrophenylphosphoryl uridine, a competitive inhibitor for RNase A. Yields were low as

compared to results reported in the literature. A purification protocol was developed to separate the inhibitor from residual reactants and side products.

Recent experiments (unpublished data) involving the DCC coupling of N-blocked amino acids and nucleosides also gave low yields with the direct coupling method. However, it was discovered that activation of the amino acids with 1-hydroxybenzotriazole before DCC coupling with the nucleosides (in the presence of 4-dimethylaminopyridine) dramatically improved yields while reducing reaction time and side products. It is felt that this activated ester approach could be applied to the synthesis of PNPU.

PNPU was reduced to the amino derivative (PAPU) and this was diazotized to produce a product that specifically coupled to the epsilon carbon of Tyr-73 when introduced into the active site of RNase A. Reduction of this compound yielded C^{ε73}-NH₂ RNase. The residual inhibitor (PAPU) was removed from the active site using affinity chromatography with PAPU as a ligand. Structure of purified C^{ε73}-NH₂ RNase was verified by IEF, UV, NMR and fluorescence spectroscopy.

Preliminary refolding experiments performed at subzero temperatures in methanolic cryosolvent at pH 3.0 and 6.0 gave a single phase with a rate constant and amplitude that compares well with data obtained by Biringer and Fink (1988a,b,c) and Puntambaeker (1992). The rate and amplitude of this transition are independent of pH. The nature of the transition characterized by this rate is currently unknown.

REFERENCES

- Anfinsen, C. *Science* 1973, 181, 223.
- Biringer, R.; *Ph.D. Thesis*, University of California at Santa Cruz, 1985.
- Biringer, R.; Fink, A. *Biochemistry* 1988a, 27, 301-310.
- Biringer, R.; Fink, A. *Biochemistry* 1988b, 27, 312-315.
- Biringer, R.; Fink, A. *Biochemistry* 1988c, 27, 315-325.
- Biringer, R.; Fink, A. *Biochemistry* 1982a, 21, 4748-4755.
- Biringer, R.; Fink, A. *J. Mol. Biol.* 1982b, 160, 87-116.
- Blum, A.; Smallcomber, S.; Baldwin, R. *J. Mol. Bio.* 1978, 118, 305-316.
- Borden, K.; Smith, M. *J. Org. Chem.* 1966, 31, 3241-3246.
- Brandts, J.; Halvorson, H.; Brennan, M. *Biochemistry*, 1975, 14, 4953.
- Chen, Y.; Fersht, A.; Hendrick, K. *J. Mol. Bio.* 1993, 234, 1158-1170.
- Cook, K.; Schmid, F.; Baldwin, R. *Proc. Natl. Acad. Sci.* 1979, 76, 6157-6161.
- Creighton, T. *Biochem. J.* 1990, 270, 1-16.
- Creighton, T. *Proteins* 1984, Chapter 4, 145, W. H. Freeman and Company, NY.
- Crook, E.; Mathias, A.; Rabin, B. 1959, 74, 234-238.
- Dill, K. *Biochemistry* 1990, 29, No.31, 7133-7155.

- Dill, K.; Fiebig, K.; Chan, H. *Proc. Natl. Acad. Sci. USA* 1993, 90, 1942-1946.
- Garel, R.; Baldwin, R. *Proc. Natl. Acad. Sci. USA* 1973, 70, 3347-3352.
- Garel, R.; Baldwin, R. *J. Mol. Bio.* 1975, 94, 621-632.
- Gehrke; Kenneth; Elsevier. *Chromatography and Modification of Nucleosides*, Part A Journal of Chromatography Library, 1991, 45A, 1190.
- Gilles, M.; Hudson, A.; Borders, Jr., C. *Analytical Biochemistry* 1990, 184, 244-248.
- Gorecki, M.; Wilchek, M. *Biochimica et Biophysica Acta*. 1978, 532, 81-91.
- Hoare, D.; Koshland, D. *J. Biol. Chem.* 1967, 242, 2447-2453.
- Karplus, M.; Weaver, D. *Nature* 1976, 260, 404-406.
- Keeton, K.; Krutzsch, H.; Lovenberg, W. *Science*, 1981, 211, 586-588.
- Khorana, H. *Chem. Rev.* 1953, 53, 145-165.
- Kim, P.; Baldwin, R. *Ann. Rev. Biochem.* 1982, 51, 459-489.
- Kuwajima, K. *Proteins: Structure, Function, and Genetics*, chapter 6, 87-103, Alan R Liss, 1989.
- Levinthal, C. *J. Chim. Phys.* 1968, 65, 44-45.
- Levitt, M. *Nature* 1975, 253, 694-698.
- Lin, t.; Brandts, J. *Biochemistry* 1983, 22, 559-573.
- Lin, T.; Koshland, D. *J. Biol. Chem.* 1969, 244, 505-508.
- Liu, W.; Tsou, C.-L. *Biochimica et Biophysica Acta* 1987, 916, 455-
-

464.

Privalov, P. *Protein Folding*, chapter 3, 83-126, W.H. Freeman and Company, NY, 1992.

Privalov, P.; Gill, S. *Adv. Protein Chem.* 1988, 39, 191.

Puntambaeker, B.; *MS Thesis*, San Jose State University, 1992.

Riehm, J.; Scheraga, H., *Biochemistry* 1965, 4, 772.

Schmid, F. *Eur. J. Biochem.* 1981, 114, 105-109.

Schmid, F. *Protein folding*, chapter 5, 197-241, W.H Freeman and Company, NY, 1992.

Schmid, F. *Biochemistry* 1983, 22, 4690-4696.

Schmid, F.; Baldwin, R. *Proc. Natl. Acad. Sci. USA* 1978, 75, 4764-4768.

Schmid, F.; Baldwin, R. *J. Mol. Bio.* 1979, 135, 199-215.

Seagle, R.; Cowgill, R. *Biochemica et Biophysica Acta* 1976, 439, 461-478.

Tsong, T.; Baldwin, R. *Biopolymers* 1978, 17, 1669-78.

Wetlaufer, D. *Proc. Nat. Acad. Sci. USA* 1973, 70, 697.

Wilchek, M.; and Gorecki, M. *Eur. j. Biochem.* 1969, 11, 491-494.

Wu, H. *Am. J. Physiol.* 1929, 90, 562.