Folding and Unfolding Kinetics of the Proline-to-Alanine Mutants of Bovine Pancreatic Ribonuclease A[†]

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ABSTRACT: Four single mutants (P42A, P93A, P114A, and P117A) of bovine pancreatic ribonuclease A (RNase A) in which each mutant has one of the four prolines of RNase A changed to alanine were prepared. The physical properties of these four mutants indicate that their native structure is essentially identical to that of wild-type RNase A. The disulfide-intact forms of these proteins were denatured in guanidine hydrochloride (Gdn.HCl) and then refolded by dilution of the Gdn.HCl. Single-jump folding, single-jump unfolding, and double-jump unfolding/folding stopped-flow experiments were carried out on wild-type and the four proline mutants of RNase A using absorption detection to follow the folding kinetics. The single-jump folding experiments carried out at six different final Gdn.HCl concentrations indicate that the folding rate constants of individual steps for the mutants are similar to those of wild-type RNase A. The Tyr92−Pro93 peptide bond has a cis conformation in native wild-type RNase A, and the results from our double-jump stopped-flow experiments indicate that the Tyr92−Ala93 peptide bond in the P93A mutant of RNase A is also cis in the native state. The existence of two cis peptide bonds (preceding Pro93 and Pro114) in wild-type RNase A is probably due to (as-yet-unidentified) long-range interactions, and such interactions are presumably the origin of a cis peptide bond even when alanine is substituted for Pro93. The data from the double-jump stopped-flow experiments are interpreted in terms of a folding/unfolding model. This model specifies the cis/trans isomerization state of the unfolded species (U_{vf}, U_f, U_m, and U_s) at *each* X−Pro peptide bond. Also, this model confirms the existence of several previously postulated chain-folding initiation sites.

The distinct reactions that proceed with different folding rates, observed when chemically denatured, disulfide-intact bovine pancreatic ribonuclease A (RNase A)[1] folds to its native[2] state, arise from a mixture of conformational isomers in the unfolded state (Garel & Baldwin, 1973; Garel et al., 1976; Schmid, 1982; Mui et al., 1985; Houry et al., 1994; Houry et al., manuscript in preparation). However, when RNase A is unfolded, and quickly refolded to the native state before any conformational isomerizations take place in the unfolded state, only a single RNase A-folding reaction is observed (Houry et al., 1994). Brandts et al. (1975) postulated that these conformationally distinct unfolded species differ in the isomeric state of the peptide bond preceding proline residues.

X−proline peptide bonds have comparable energies in both the cis and trans state (Zimmermann & Scheraga, 1976), while other peptide bonds exist almost exclusively in the trans conformation. In the unfolded state of a protein, the X−proline peptide bonds may exist in either the cis or trans isomeric states, usually a mixture of both. However, in the native state of a protein, the conformation is generally fixed in either the cis or trans state. Therefore, when an unfolded protein with a mixture of cis and trans states for an X−proline peptide bond folds to the native state, unfolded protein molecules with the X−proline peptide bond in the isomeric state of the native protein may be expected to fold at a faster rate than those molecules with a non-native X−proline peptide bond isomeric state.

Since a polypeptide chain cannot possibly search all conformations when folding from a denatured state to a folded state, it must follow one or more pathways to the native state. A polypeptide will initially form structure at some sites, thus limiting the number of conformations possible. Identifying the sites where structure initially forms, called chain-folding initiation sites (CFIS's) (Matheson & Scheraga, 1978; Montelione & Scheraga, 1989), will help in understanding how a polypeptide folds from an unfolded state to the native state of a protein.

Proline residues can act as probes for CFIS's in proteins. Since the unfolded state of a protein has a mixture of both cis and trans isomers of the X−proline peptide bonds, only those protein molecules that have the native X−proline peptide bond isomers or that have the non-native X−proline peptide bond isomers in regions that are not CFIS's will fold very fast. Thus, by examining which proline residues affect

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; P42A, P93A, P114A, and P117A RNase A, proline-to-alanine mutants of ribonuclease A; U_{vf}, U_f, U_m, U_s^{II}, and U_s^{I}, very fast-, fast-, medium-, and two slow-folding conformationally distinct unfolded species of RNase A, respectively; cCMP, cytidine 2′,3′-cyclic phosphate; EDTA, disodium ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Gdn.HCl, guanidine hydrochloride; Gdn.SCN, guanidine thiocyanate; GSH, reduced glutathione; GSSG, oxidized glutathione; IPTG, isopropyl β-D-thiogalactoside; NTB, 2-nitro-5-thiosulfobenzoate; NTSB, disodium 2-nitro-5-thiosulfobenzoate; 2XYT LB, two times yeast−tryptone liquid broth; wt, wild-type.

[2] The term "native" is used throughout this paper to refer to the completely folded wild-type and mutant proteins.

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the folding of a protein, we can identify whether regions around the prolines may or may not be CFIS's.

The cis/trans isomerization of proline residues in proteins results in a distribution of unfolded species, with each unfolded species having different folding rates. The difference in folding rates between the fastest-folding species and the slowest-folding species of a protein may be several orders of magnitude. If each of the unfolded species differs by only the cis/trans isomeric state of the peptide bonds preceding proline, it would be useful to know why the same isomerization reaction occurring at different places in the protein (different proline residues) results in such different folding rates. A detailed model describing which proline residues are responsible for the various unfolded species will help in understanding protein-folding pathways in general.

Bovine pancreatic ribonuclease A has proline residues at positions 42, 93, 114, and 117. The Lys41−Pro42 and Val116−Pro117 peptide bonds are trans in the native state, while the Tyr92−Pro93 and Asn113−Pro114 peptide bonds are cis (Wlodawer, 1980). We have prepared four mutants of RNase A in which each of the four mutants has one proline replaced by alanine. The disulfide-intact unfolding and refolding kinetics of these four mutants of RNase A have been investigated, and a model is developed to account for the various unfolded species in wild-type RNase A. From this model, we find further support for the location of the CFIS's in RNase A.

MATERIALS AND METHODS

Construction of a Plasmid for Expression of Proline Mutants of RNase A. A synthetic gene for bovine ribonuclease A was a gift from the Genex Corp. This gene was placed in M13mp18 (United States Biochemical) (Laity et al., 1993), and mutagenesis was carried out using the T7-Gen in vitro mutagenesis kit (United States Biochemical). The codons in the synthetic gene for the −2 and −1 amino acids were changed to those for methionine and alanine, respectively, in order to create an *Msc*I restriction enzyme site for insertion of this gene into the expression plasmid. Four mutants, P42A, P93A, P114A, and P117A, of this gene were created in which, in each one, the codon for one of the four prolines in RNase A was changed to GCT (alanine). The mutagenesis was confirmed by DNA sequencing at the Cornell Biotechnology Analytical Facility.

delCardayré et al. (1995) have reported high yields of RNase A using pET22b(+). Therefore, the proline mutants of RNase A in M13mp18 were cloned into pET22b(+) (Novagen) between the *Msc*I and *Hin*dIII restriction enzyme sites. The plasmids containing the proline mutants P42A, P93A, P114A, and P117A were transformed into the *Escherichia coli* cell line BL21(DE3) (Novagen) which was stored at −70 °C.

Protein Expression. The protein expression system used for developing folding conditions to form the disulfide bonds from the reduced protein isolated from *E. coli* was that of Laity et al. (1993). The procedure for expressing each of the four proline mutants used in the disulfide-intact folding studies was identical and is descibed here. The frozen cells were streaked on a 50 μg/μL ampicillin agar plate, and the cells were grown overnight at 37 °C. Then, a single colony of BL21(DE3) containing a proline mutant in pET22b(+) from the plate was grown for an additional 5 h in 3 mL of 2XYT LB medium with 50 μg/μL ampicillin at 37 °C. After 5 h, 1 mL of this culture was added to 1 L of the same medium and grown at 37 °C until the optical density at 570 nm reached 0.8. At this time, protein expression was induced by addition of 2 mL of 0.5 mM IPTG to 1 L of cells. These cells were pipetted onto various plates, and the number of colonies on an ampicillin agar plate was compared to the number of colonies on an IPTG agar plate to determine the viability of the cells in the presence of expressed RNase A; it was determined that greater than 99% of the *E. coli* cells carry the pET22b(+) plasmid prior to induction. After induction, the cells were shaken at 37 °C for 3 h. After 3 h, the cells were centrifuged and resuspended in 20 mL of 100 mM NaCl. The cells were sonicated for 5 min with 2 s pulses to lyse the cells and break up the genomic DNA. The lysed cells were centrifuged and resuspended in 5 mL of 4 M guanidine thiocyanate (Gdn.SCN), 2 mM EDTA, 100 mM Tris, and 80 mM reduced glutathione (GSH) at pH 8.0. This solution was stirred for 2 h to solubilize the RNase A. The insoluble cell material was centrifuged, and the soluble portion was diluted with 200 mL of 100 mM Tris and 2 mM EDTA at pH 8.0. This results in a solution that is 2 mM EDTA, 100 mM Tris, 2 mM GSH, 100 mM Gdn.SCN, and approximately 15 μM disulfide-reduced RNase A. Formation of the four disulfide bonds of RNase A was accomplished by addition of 62 mg of oxidized glutathione (GSSG) to the solution of reduced RNase A. The folding solution, which was 2 mM GSH and 0.5 mM GSSG at pH 8.0, was stirred for 72 h at 4 °C.

Protein Purification. As reported previously (Schultz et al., 1992), the ribonuclease A mutants P93A and P114A are less soluble than wild-type RNase A. In addition, only about 60% of the reduced P93A and P114A RNase A molecules had formed the four native disulfide bonds, while the remainder of the molecules either had only three disulfides or were insoluble aggregates of unknown composition.

P42A, P114A, and P117A were purified on a preparative Hydropore cation exchange column (Rainin). The pH of the folding solution was lowered to 5 with acetic acid and the solution filtered. The volume of this solution was reduced using an ultrafiltration cell (Amicon) with a YM3 membrane. This solution was loaded onto the preparative Hydropore cation exchange column (Rainin), and using a buffer of 25 mM Hepes and 1 mM EDTA at pH 7, a linear gradient from 0 M to 20 mM NaCl was run. Wild-type and these three proline mutants of RNase A with the four correct disulfide bonds appear as the last peak to elute from this cation exchange column. Positive identification of this peak as native RNase A is proven by the physical characterization reported in the Results.

P93A RNase A is not soluble enough to be purified by the above method. P93A RNase A is soluble in 4 M urea at pH 4; however, purification on a Rainin Hydropore preparative cation exchange column using a linear gradient of NaCl results in multiple species (molecules with only three disulfides, deamidated RNase A, as well as native P93A RNase A) eluting at the same salt concentration. It was, therefore, necessary to carry out the purification of P93A RNase A at pH 7.

As the pH of a urea solution increases, the equilibrium between urea and ammonium cyanate shifts. At pH 7, the concentration of ammonium cyanate can become significant. Ammonium cyanate will react with RNase A to carbamylate

lysine residues irreversibly (Stark et al., 1960). Therefore, urea must not be kept at pH 7 for long times to minimize the formation of ammonium cyanate. This was accomplished by purification of P93A RNase A with the following high-performance liquid chromatography (HPLC) ternary gradient. Buffer A was 100 mM Hepes, 1 mM EDTA at pH 7.3. Buffer B was 100 mM Hepes, 1 mM EDTA, and 1 M NaCl at pH 7.3. Buffer C was 20 mM formic acid and 6 M urea at pH 4. The gradient used to purify P93A was 80% C and a linear change from 20% A and 0% B to 0% A and 20% B. This resulted in purified P93A RNase A, while urea was exposed to pH 7 only for the length of the gradient (30 min). After P93A eluted from the cation exchange column, the pH was again adjusted to pH 4. Since carbamylation of the lysine residues reduces the charge on the protein, any carbamylation can be observed on the cation ion exchange column. Using this method of purification, no detectable amount of carbamylation was observed.

Wild-type RNase A Type 1-A (Sigma) was purified by ion exchange chromatography (Rothwarf & Scheraga, 1993).

All proteins were desalted after ion exchange chromatograpy purification using an ultrafiltration cell (Amicon). The buffers were exchanged with a 30/70 acetonitrile/water solution in which the proline mutants of RNase A are soluble. The proteins, with less than 1% salt remaining, were then lyophilized, resulting in pure, dry RNase A mutants that were used in subsequent experiments. Amino acid analysis and laser desorption mass spectrometry, carried out by the Cornell Biotechnology Analytical Facility, confirmed the correct composition of the proline mutants.

Extinction Coefficient Determination. The extinction coefficients of wild-type and the four proline mutants of RNase A were determined by using the method of Thannhauser et al. (1987). The absorbance of a solution of RNase A (approximately 50 μM) in 100 mM acetic acid was measured at 275 nm. The molarity of disulfide bonds of the same solution was determined by addition of 50 μL to 2300 μL of 2 M Gdn.SCN, 50 mM glycine, 100 mM sodium sulfite, 3 mM EDTA, and 25 mM NTSB at pH 9.5. One mole of disulfide bonds reacts quantitatively with 1 mol of NTSB to form 1 mol of NTB. Formation of NTB was monitored at 412 nm by using its extinction coefficient of 13 900 M^{-1} cm^{-1} (Thannhauser et al., 1987). Since there are four disulfide bonds in RNase A, the molarity of native protein can be determined; dividing the absorbance obtained at 275 nm by the molarity of the protein solution then gives the extinction coefficient at 275 nm.

Enzyme Activity. RNase A activity measurements were made at pH 5 and 22 °C using cytidine 2′,3′-cyclic phosphate (cCMP) (Sigma) as a substrate (Crook et al., 1960). The slopes (Δabsorbance/Δtime) at 286 nm for four concentrations [$(0.5-10) \times 10^{-8}$ M] of protein were compared to the slope divided by concentrations for wild-type RNase A, and the activities of the mutants are reported as percent activity of wild-type RNase A.

Melting Temperature. The T_m's of wild-type and the four proline mutants were determined under several conditions. Protein concentration was approximately 3×10^{-5} M for all thermal transitions. The melting temperatures were determined in 40 mM glycine and 0.2 M guanidine hydrochloride (Gdn.HCl) at pH 3.0, in 50 mM formic acid and 0.2 M Gdn.HCl at pH 4.0, in 50 mM sodium acetate and 0.2 M Gdn.HCl at pH 5.0, and in 50 mM sodium acetate and 1.3 M Gdn.HCl at pH 5. Gdn.HCl concentrations were determined by refractive index (Pace et al., 1989). Protein in the buffer was placed in a 1 mL quartz cuvette in a modified Cary 14 spectrophotometer (Denton et al., 1982). A calibrated thermistor was also placed in the sample compartment. A matched quartz cuvette with buffer only was placed in the reference chamber. Using a program written in this laboratory to control a Neslab RTE-100 circulation bath and to record the temperature of the sample chamber through the thermistor, the temperature of the protein was raised and lowered through the melting point while the absorbance was monitored at 287 nm. Reversibility of the folding and unfolding of the protein through the transition was ensured by the reversibility of the thermal transition curves. The melting temperature was determined by fitting a straight line to points in the pre- and post-transition regions and extrapolating to the midpoint where 50% of the protein molecules are folded and 50% are unfolded.

Guanidine Hydrochloride Transitions. Aliquots of protein were added to solutions of 100 mM sodium acetate at pH 5, and various concentrations of Gdn.HCl, to yield final solutions with approximately 20 μM protein. Gdn.HCl concentrations were determined with a Baush and Lomb refractometer (Pace et al., 1989). Solutions were allowed to equilibrate for $\geq$2 h at 15 °C before their absorbance at 287 nm was measured in a 1 mL quartz cuvette. Midpoints of the transitions were determined by using the linear extrapolation method described by Santoro and Bolen (1988).

Stopped-Flow Kinetic Experiments. Experiments to measure the folding rate of Gdn.HCl-denatured proteins were carried out on a Hi-Tech PQ/SF-53 stopped-flow apparatus. UV radiation from a deuterium lamp (Hellma) was passed through a calibrated monochrometer at 287 nm and then through a 10 mm flow cell containing protein solution to a photomultiplier tube. The signal from the photomultiplier tube was collected with Soft500 software (Keithly). Data points, each averaged over 330 μs, were collected every 0.5 ms for the first 60 s after folding was initiated and then every 50 ms for up to 20 min after folding was initiated. All measurements were made at 15 °C.

Single-jump folding experiments were carried out by mixing 11 μL of 4.2 M Gdn.HCl, 40 mM glycine, and 800 μM RNase A with 109 μL of 50 mM sodium acetate at pH 5.3 containing Gdn.HCl at various concentrations. The concentrations of Gdn.HCl in the folding buffer (prior to mixing) were 0.01, 0.24, 0.44, 0.66, 0.87, or 1.07 M to give final folding conditions of 4 mM glycine, 45 mM sodium acetate, pH 5, and 0.4, 0.6, 0.8, 1.0, 1.2, or 1.3 M Gdn.HCl. Folding experiments at each final Gdn.HCl concentration were repeated at least five times.

Single-jump unfolding experiments were carried out by mixing 25 μL of 40 mM sodium acetate at pH 5, 1.3 M Gdn.HCl, and 250 μM RNase A with 125 μL of 60 mM glycine at pH 1.7 and 4.4 M Gdn.HCl to give final unfolding conditions of 42 μM RNase A, 3.9 M Gdn.HCl, and pH 2. Measurements were repeated at least five times.

Double-jump folding experiments were carried out by mixing 52 μL of 40 mM sodium acetate at pH 5, 1.3 M Gdn.HCl, and 1500 μM RNase A with 128 μL of 60 mM glycine at pH 1.5 and 5.1 M Gdn.HCl to give an unfolding solution that was 3.9 M Gdn.HCl at pH 2 and 428 μM RNase A. After a delay time, which varied over the range 0.2−

Table 1: Properties of Wild-Type and Mutant RNase A's

	WT	P42A	P93A	P114A	P117A
extinction coefficient[a] (M^{-1} cm^{-1})	9700 (200)	9900 (200)	11 770 (400)	10 800 (200)	10 000 (200)
activity[b] (%)	100 (6)	57 (6)	69 (7)	82 (9)	83 (9)

[a] At 275 nm in 100 mM acetic acid. Numbers in parentheses are the standard deviations. [b] Relative to wild-type RNase A, against cCMP at pH 5 at 22 °C. Numbers in parentheses are the standard deviations.

600 s, during which the protein was unfolded, folding was then initiated by mixing 53 μL of this unfolded protein with 267 μL of 100 mM sodium acetate at pH 5.2 and 0.8 M Gdn.HCl to give final folding conditions of 71 μM RNase A, pH 5.0, and 1.3 M Gdn.HCl. Folding at each delay time was repeated at least three times.

Analysis of Kinetic Data. The following equation was used to fit the time dependence of the changes in tyrosine absorbance during folding and unfolding.

$$A(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + ...a_n e^{-t/\tau_n} + C \qquad (1)$$

In this equation, $A(t)$ is the absorbance at time t, τ is the time constant of a phase, a is the amplitude of the phase, n is the number of exponentials required to fit the observed absorbance change upon folding or unfolding adequately, and C is a constant. The experimental data were fit to this equation by using the Levenberg−Marquardt algorithm to minimize χ^2 (Press et al., 1990). Depending on the folding conditions, RNase A may have as many as four distinct folding phases, requiring a four-exponential equation to fit the data. The following procedure was used to fit the data from the single-jump folding and single-jump unfolding experiments.

The data from 20 s to 10 or 15 min were fit to either a single or, if necessary, a double exponential. Then, if these exponentials were kept fixed, the data were fit from 20 to 300 ms to an additional exponential. Then, if the two or three exponentials were kept fixed, the data were fit, if necessary, to an additional exponential between 20 ms and 10 s. Determining four time constants in data from 0 to 600 s may not be accurate because of the possibility that several sets of time constants could result in equally good fits to the experimental data; however, since all data sets were treated identically, comparison of the rates and amplitudes is possible.

The data from the double-jump refolding experiments were fitted as follows. The single fast-folding species observed with a short unfolding time (delay time) of 0.2 s was fit to a single exponential to obtain the time constant for this folding species. The reason for the adoption of this procedure was that, after long unfolding times, this unfolded species had an amplitude too small to be observed. Time constants for the other unfolded species were taken from the single-jump folding experiments at the same final Gdn.HCl conditions. The time constants were kept fixed, and the amplitudes of the unfolded species at the various delay times were determined by fitting the data from the double-jump folding experiments.

The differential equations, describing the rates of formation of the various unfolded species in the double-jump folding experiments, were solved numerically using a fourth order Runge−Kutta algorithm. The best values for the rate constants (k^1−k^7) were obtained by varying them with a simplex algorithm (Press et al., 1990) to obtain values that best fit the experimental data from all four double-jump folding experiments simultaneously.

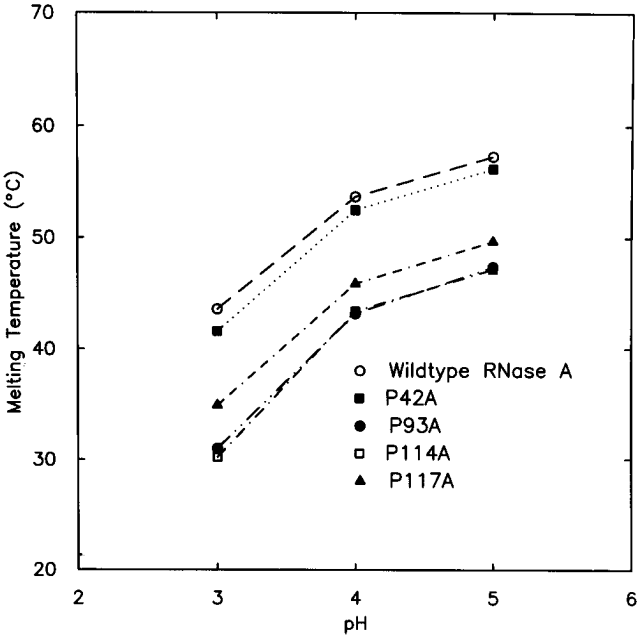


FIGURE 1: Melting temperatures of wild-type and each of the four proline mutants of RNase A at pH 3, 4, and 5. Straight lines connect the points to aid in interpretation.

RESULTS

Protein Characterization. The extinction coefficients and relative activities of wild-type and the four proline mutants of RNase A are listed in Table 1. The similarity of the activities indicates that all four mutants have native structures close to that of wild-type RNase A. The proline 93 and proline 114 mutants of RNase A have extinction coeffcients significantly different from that of wild-type RNase A. This is not surprising since residues 92 and 115 in RNase A are tyrosine residues. Therefore, local changes in structure around residues 93 and 114 may be expected to cause a change in the absorbance of tyrosine residues 92 and 115, respectively.

The melting temperatures of wild-type and the four proline mutants at several pH's and 0.2 M Gdn.HCl are shown in Figure 1. The reversibility of the transitions and consistent changes in stability with pH strongly suggests that, under these conditions, the proline mutants of RNase A do not aggregate and are completely soluble. It was not possible, however, to obtain reversible thermal transitions with the proline mutants of RNase A at pH 7, suggesting that aggregation may occur at a pH higher than 5.

The fact that the four proline mutants of RNase A have reversible thermal transitions and relative activities against cCMP similar to those of wild-type RNase A indicates that they have a native structure and their native structure is similar to that of wild-type RNase A. RNase A with only

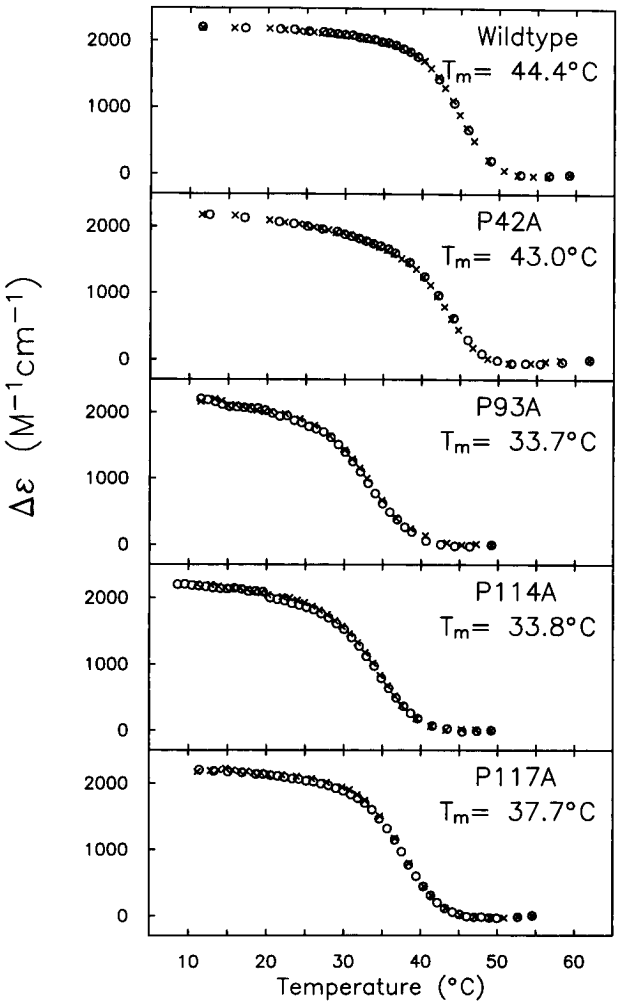


FIGURE 2: Thermal transition curves for wild-type and each of the four proline mutants of RNase A at 1.3 M Gdn.HCl and pH 5 measured by absorbance at 287 nm. The heating (○) and cooling (×) curves are plotted.

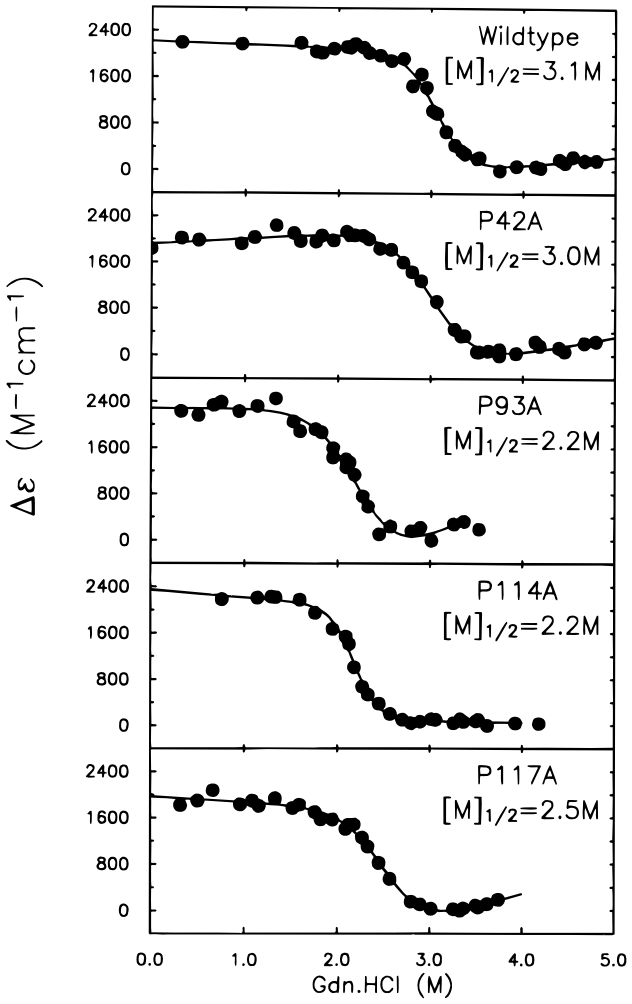


FIGURE 3: Guanidine hydrochloride transition curves for wild-type and each of the four proline mutants of RNase A at pH 5 and 15 °C measured by absorbance at 287 nm. The solid line is the fit to the free energy equation described in the text.

three of the four native disulfide bonds has native structure and some activity against cCMP (Li et al., 1995); hence, formation of the four disulfides of native RNase A is confirmed by the following experimental results. Folding from the disulfide-reduced to the four-disulfide native RNase A was accomplished by using glutathione as the redox agent. The cysteine residues in RNase A form intermolecular disulfide bonds with glutathione. Since each intermolecular disulfide bond with glutathione will reduce the positive charge, at pH 7, of RNase A by 1, any RNase A molecules having less than four intramolecular disulfide bonds will elute before native RNase A from the cation exchange column. In addition, the mass of the proline mutants of RNase A in their native state, determined by laser desorption mass spectrometry, was the same as expected for native RNase A and less than the mass of RNase A with intermolecular disulfide bonds with glutathione.

Figure 2 shows thermal transitions for wild-type and the four proline mutants of RNase A at pH 5 and 1.3 M Gdn.HCl (i.e., at a higher Gdn.HCl concentration than in Figure 1). The highest Gdn.HCl concentration used in the folding experiments is 1.3 M. All the proline mutants are completely folded at 1.3 M Gdn.HCl, 15 °C, and pH 5. Figure 3 shows the Gdn.HCl transition curves for wild-type and the four proline mutants of RNase A. As in the thermal transition experiments in Figures 1 and 2, P93A and P114A RNase A have the same stability, P117A RNase A is slightly more stable, and P42A and wild-type RNase A have nearly the same (higher) stability.

Single-Jump Folding. The results from the single-jump folding experiments followed by absorbance at 287 nm are shown in Tables 2−5. The results are also shown graphically to aid in interpretation in Figures 4−6. The results for folding wild-type RNase A are consistent with those reported in the literature. At 0.4 M Gdn.HCl, the ratio of the amplitudes of the two slow-folding species, U_s^{II} to U_s^{I}, is approximately 80/20. A fast-folding species, U_f, is also observed with 14% of the total amplitude. The very fast-folding species, U_{vf}, is not observed in this experiment because of its small amplitude. The medium-folding species, U_m, is observed with a folding amplitude of 11% of the total amplitude. As the final Gdn.HCl concentration increases, the folding rates of the unfolded species decrease while their relative amplitudes remain the same. Because the folding kinetics of the U_m species have a small total amplitude, it is not possible to determine the Gdn.HCl dependence of the folding rate. We have therefore assumed that the folding rate of the U_m species depends on the Gdn.HCl concentration. At 1.2 and 1.3 M Gdn.HCl, the folding rates of the two slow-folding species of wild-type RNase A become too close to be separated into two distinct phases and thus are observed as a single slow-folding species.

Table 2: Relative Amplitudes and Time Constants for the Refolding of the Fast-Folding Species ($U_{vf} + U_f$) of Wild-Type and the Four Proline Mutants of Ribonuclease A at Several Final Gdn.HCl Concentrations at pH 5 and 15 °C[a]

final Gdn.HCl (M)	WT	P42A	P93A	P114A	P117A
			Amplitudes (%)		
0.4	14	19	0[b]	21	17
0.6	14	21	0	15	18
0.8	16	21	0	19	16
1.0	18	21	0	18	17
1.2	15	19	0	19	16
1.3	14	13	0	20	15
			Time Constants (ms)		
0.4	49	44	–	29	38
0.6	49	49	–	40	44
0.8	76	50	–	51	52
1.0	90	73	–	79	63
1.2	120	99	–	87	99
1.3	186	130	–	125	167

[a] These results are shown graphically in Figure 4. [b] No fast-folding species was observed for P93A RNase in these experiments.

Table 3: Relative Amplitudes and Time Constants for the Refolding of the Medium-Folding Species (U_m) of Wild-Type and the Four Proline Mutants of Ribonuclease A at Several Final Gdn.HCl Concentrations at pH 5 and 15 °C[a]

final Gdn.HCl (M)	WT	P42A	P93A	P114A	P117A
			Amplitudes (%)		
0.4	11	9	13	7	12
0.6	13	10	13	15	12
0.8	10	14	11	9	10
1.0	9	7	12	5	9
1.2	10	9	10	7	10
1.3	9	11	9	7	11
			Time Constants (s)		
0.4	1.3	1.8	1.1	2.4	2.2
0.6	2.3	2.5	1.8	4.5	1.8
0.8	2.3	3.3	1.6	3.7	1.8
1.0	2.8	2.2	2.7	3.0	1.9
1.2	3.0	1.8	3.1	2.5	1.8
1.3	2.1	1.4	3.6	2.0	2.1

[a] These results are shown graphically in Figure 5.

Table 4: Relative Amplitudes and Time Constants for the Refolding of the Slow-Folding Species (U_s^{II}) of Wild-Type and the Four Proline Mutants of Ribonuclease A at Several Final Gdn.HCl Concentrations at pH 5 and 15 °C[a]

final Gdn.HCl (M)	WT	P42A	P93A	P114A	P117A
			Amplitudes (%)		
0.4	57	60	73	52	70[b]
0.6	59	59	71	47	70
0.8	59	55	71	33	74
1.0	55	61	71	18	74
1.2	75	72	66	7	74
1.3	76	76	58	0	75
			Time Constants (s)		
0.4	19	19	7	13	48[b]
0.6	30	28	10	23	75
0.8	47	35	17	29	117
1.0	70	70	32	26	168
1.2	134	126	55	43	221
1.3	173	166	87	–	240

[a] The results are shown graphically in Figure 6. [b] Only a single U_s-folding species was observed for P117A RNase A.

The proline 42-to-alanine 42 mutant of RNase A shows behavior nearly identical to that of wild-type RNase A. The small difference in the folding rate and relative amplitudes of the unfolded species does not indicate any significant change in the folding pathway.

Table 5: Relative Amplitudes and Time Constants for the Refolding of the Slow-Folding Species (U_s^I) of Wild-Type and the Four Proline Mutants of Ribonuclease A at Several Final Gdn.HCl Concentrations at pH 5 and 15 °C[a]

final Gdn.HCl (M)	WT	P42A	P93A	P114A	P117A
			Amplitudes (%)		
0.4	18	12	14	20	0[b]
0.6	15	10	16	23	0
0.8	15	10	18	39	0
1.0	15	10	18	59	0
1.2	0	0	25	67	0
1.3	0	0	32	73	0
			Time Constants (s)		
0.4	81	107	93	80	–
0.6	127	186	138	116	–
0.8	171	182	142	113	–
1.0	181	236	183	148	–
1.2	–	–	197	235	–
1.3	–	–	219	306	–

[a] The results are shown graphically in Figure 6. [b] Only a single U_s-folding species was observed for P117A RNase A.

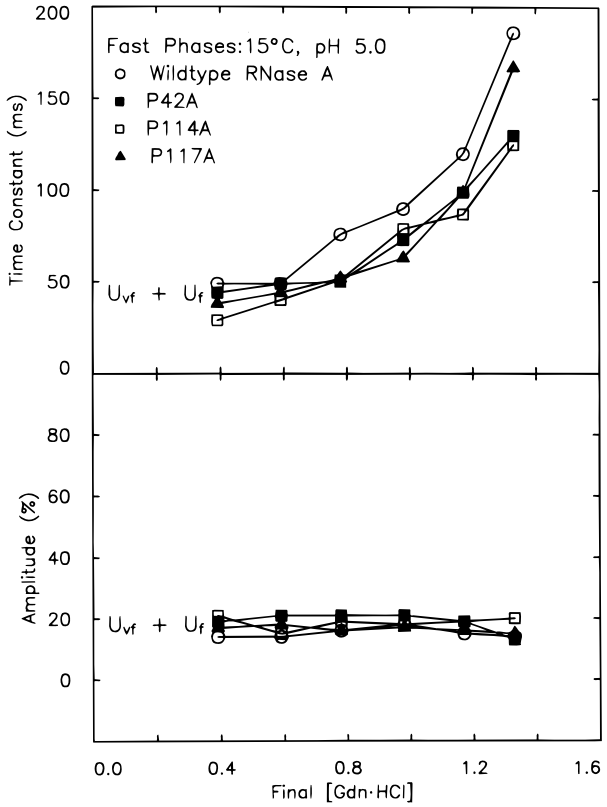


FIGURE 4: Plot of the amplitudes and time constants at several final Gdn.HCl concentrations for the fast-folding species of wild-type and three proline mutants of RNase A.

The slow-folding species of the proline 93-to-alanine 93 mutant of RNase A behave in a manner similar to that of the slow-folding species of wild-type RNase A. The ratio of the amplitudes of the two species, U_s^{II} to U_s^I, is approximately 80/20, and they have time constants similar to those of wild-type RNase A. A medium-folding species, U_m, is also observed. However, no fast-folding species, U_f, is observed, and the medium-folding species, U_m, is the fastest-folding species of P93A RNase A observed. At 1.2 and 1.3 M Gdn.HCl, the ratio of the amplitudes of the U_s^{II} to U_s^I folding species is no longer 80/20. This is most likely due to the fact that the two species, U_s^{II} and U_s^I, have similar

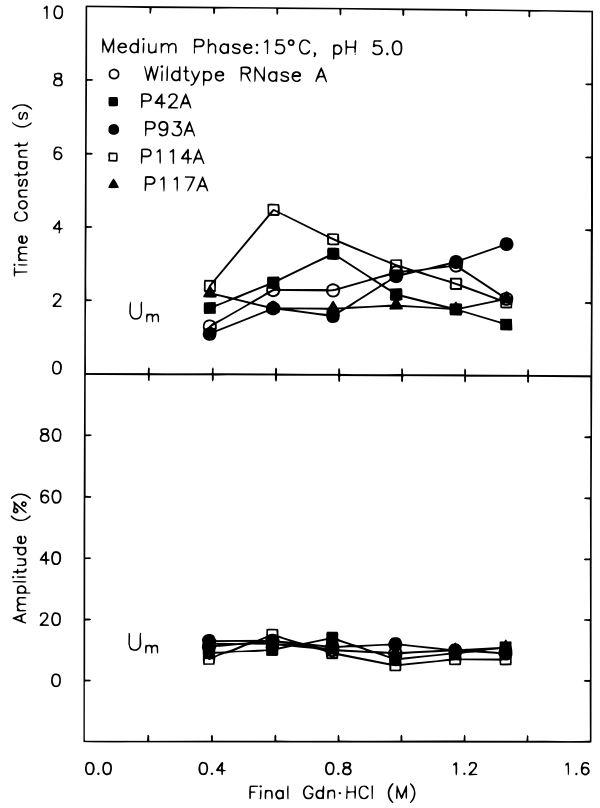


FIGURE 5: Plot of the amplitudes and time constants at several final Gdn.HCl concentrations for the medium-folding species of wild-type and the four proline mutants of RNase A.

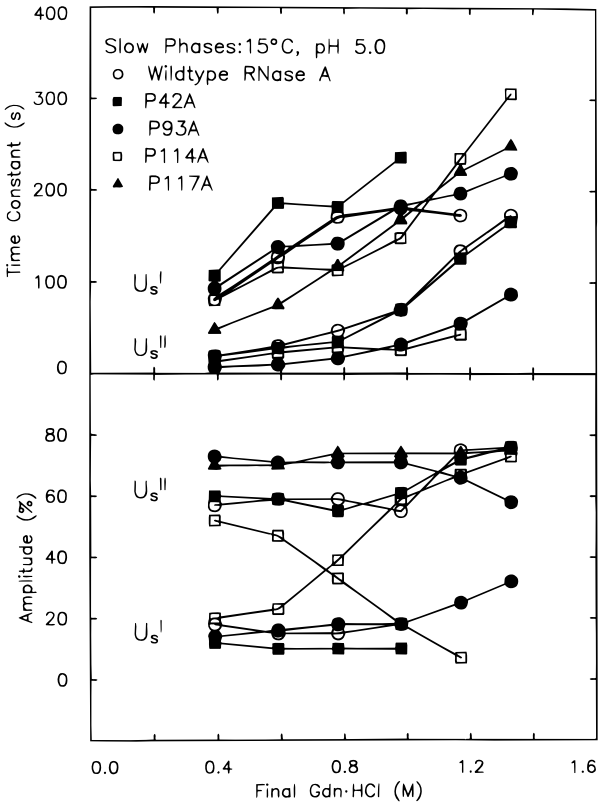


FIGURE 6: Plot of the amplitudes and time constants at several final Gdn.HCl concentrations for the slow-folding species of wild-type and the four proline mutants of RNase A

time constants and, at the higher Gdn.HCl concentrations, it is not possible to fit them accurately as two different species.

Table 6: Time Constant for the Single Unfolding Phase of Wild-Type and the Four Proline Mutants of RNase A under Final Unfolding Conditions of 3.9 M Gdn.HCl, pH 2, and 15 °C

	WT	P42A	P93A	P114A	P117A
τ (ms)	68	59	37	33	48

The slow-folding species of the proline 114-to-alanine 114 mutant of RNase A do not behave like the slow-folding species of wild-type RNase A. The time constants for folding of the U_s^I and U_s^{II} species of P114A are similar to those of wild-type RNase A; however, the relative amplitudes of the two slow-folding species change with the increase in the final Gdn.HCl concentration, while the relative amplitudes of the two slow-folding species of wild-type RNase A stay constant with the increase in the final Gdn.HCl concentration. The U_m and U_f species of P114A RNase A are very similar in amplitude and rate to those of wild-type RNase A.

The proline 117-to-alanine 117 mutant of RNase A has only one slow-folding species, U_s, at all the Gdn.HCl concentrations where folding was measured. The time constant for the folding of this species is between the time constants of U_s^I and U_s^{II} of wild-type RNase A. The medium- and fast-folding species of P117A RNase A are very similar to those of wild-type RNase A.

Single-Jump Unfolding. The unfolding rates for wild-type and the four proline mutants of RNase A were determined by monitoring the absorbance change when native protein in 1.3 M Gdn.HCl at pH 5 was unfolded at 3.9 M Gdn.HCl, pH 2, and 15 °C. The wild-type and mutant proteins are completly unfolded under these conditions (data not shown). A single unfolding phase was observed for wild-type and the four proline mutants of RNase A. The time constants for unfolding are listed in Table 6.

Double-Jump Folding. Double-jump unfolding experiments were carried out on wild-type, P93A, P114A, and P117A RNase A. The protein was unfolded from the native state at 1.3 M Gdn.HCl and pH 5, to the unfolded state at 3.9 M Gdn.HCl, pH 2, and 15 °C for various delay times between 0.2 and 600 s. Then, the protein was refolded back to the native state at 1.3 M Gdn.HCl and pH 5.

Houry et al. (manuscript in preparation) have found that, under favorable folding conditions (low Gdn.HCl concentrations), it is not possible to distinguish between the U_{vf} and U_f folding species. However, under unfavorable folding conditions (high Gdn.HCl concentrations), the U_{vf} and U_f folding species can be resolved as two distinct species. We have, therefore, carried out our double-jump folding experiments at a final Gdn.HCl concentration of 1.3 M where the U_{vf} and U_f folding species can be observed as two distinct species. When folding at a final high Gdn.HCl concentration of 1.3 M, the U_s^I and U_s^{II} folding species of wild-type and the proline mutants of RNase A cannot be resolved as two distinct species as seen in the single-jump folding experiments. In the double-jump folding experiments at 1.3 M Gdn.HCl, we therefore refer to the U_s^I and U_s^{II} species collectively as U_s since we are not able to distinguish between them. The relative amplitudes of the various unfolded species were determined during refolding after unfolding for a given time, and these results are shown in Figures 7−10.

Wild-type RNase A, unfolded for 600 s (Figure 7), has three observable folding species, U_s, U_m, and U_f, as seen in

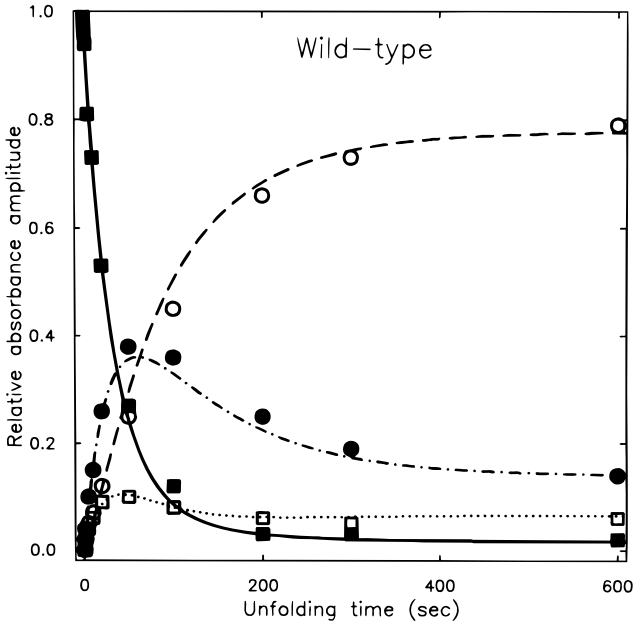


FIGURE 7: Relative amplitudes versus time of unfolding of the U_{vf} (■), U_f (●), U_m (□), and U_s (○) species of wild-type RNase A at final folding conditions of 1.3 M Gdn.HCl, pH 5, and 15 °C. The solid curves are fits to the proposed kinetic model in eq 2 with the rate constants listed in Table 7.

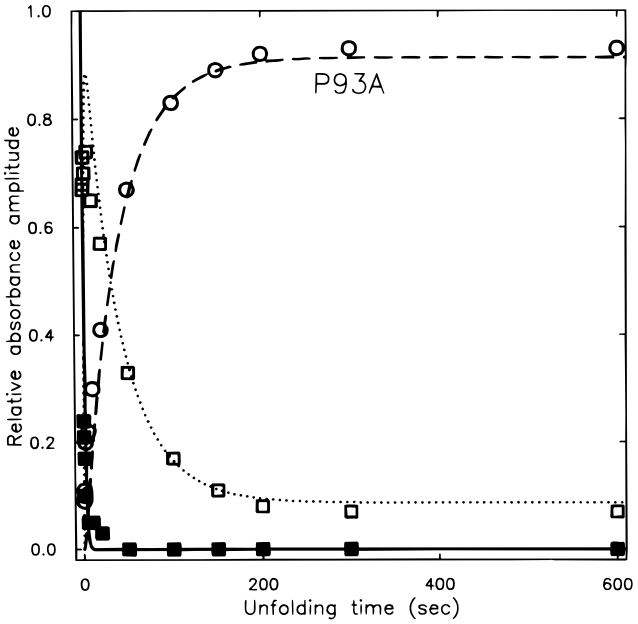


FIGURE 8: Relative amplitudes versus time of unfolding of the U_{vf} (■), U_m (□), and U_s (○) species of P93A RNase A at final folding conditions of 1.3 M Gdn.HCl, pH 5, and 15 °C. The solid curves are fits to the proposed kinetic model in eq 3 with the rate constants listed in Table 7.

the single-jump folding experiments under the same final folding conditions. After the enzyme was unfolded for 0.2 s before being refolded, isomerizations, such as the cis to trans isomerization of an X−proline peptide bond, do not have time to occur to a great extent. Therefore, only one unfolded species, U_{vf} (Houry et al., 1994), was observed after wild-type RNase A was unfolded for 0.2 s. The time constant for the folding of this species was 66 ms. At unfolding times longer than 0.2 s, the other unfolded species, observed in the single-jump folding experiments, begin to appear. The solid lines in Figure 7 are the fit of the data

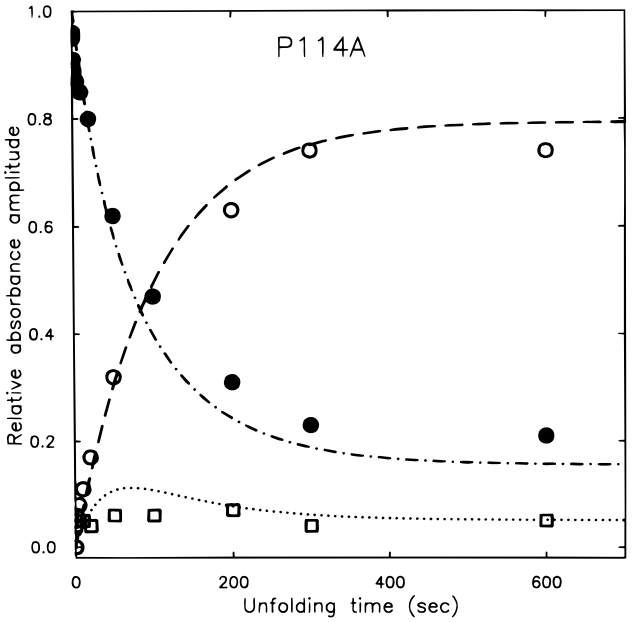


FIGURE 9: Relative amplitudes versus time of unfolding of the U_f (●), U_m (□), and U_s (○) species of P114A RNase A at final folding conditions of 1.3 M Gdn.HCl, pH 5, and 15 °C. The solid curves are fits to the proposed kinetic model in eq 4 with the rate constants listed in Table 7.

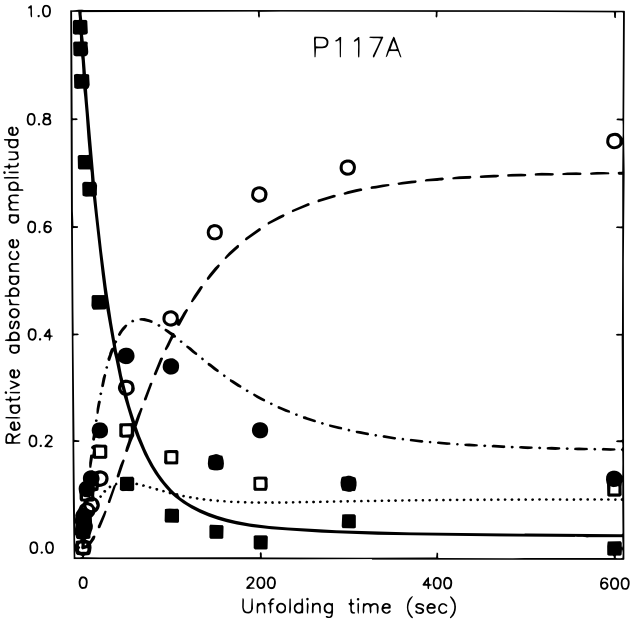
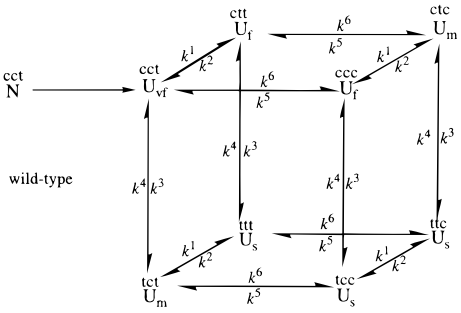


FIGURE 10: Relative amplitudes versus time of unfolding of the U_{vf} (■), U_f (●), U_m (□), and U_s (○) species of P117A RNase A at final folding conditions of 1.3 M Gdn.HCl, pH 5, and 15 °C. The solid curves are fits to the proposed kinetic model in eq 5 with the rate constants listed in Table 7.

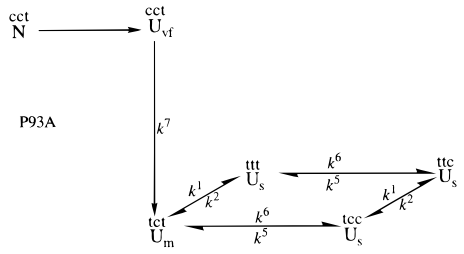
points to the kinetic model in eq 2.



(2)

In this equation, N represents the native protein and U_{vf}, U_f, U_m, and U_s represent the various unfolded species. The three letters (c or t) above the various unfolded species represent the cis (c) or trans (t) isomeric state of the peptide bond preceding residues 93, 114, and 117, respectively. Wild-type RNase A has the native conformation of cis 93, cis 114, and trans 117 (cct). The rate constants k^1, k^3, and k^5 pertain to the cis to trans isomerizations of the peptide bond preceding residues 114, 93, and 117, respectively. The rate constants k^2, k^4, and k^6 pertain to the trans to cis isomerizations of the peptide bonds preceding residues 114, 93, and 117, respectively. The rationale for the assignment of the various unfolded species to the peptide bond isomeric states is described in the Discussion.

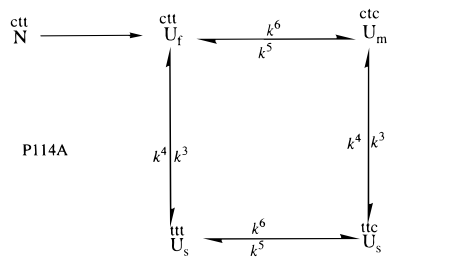
The results of the double-jump folding experiment on P93A RNase A are shown in Figure 8. At an unfolding time of 600 s, there are three refolding phases as seen in the single-jump folding experiments under the same final folding conditions. At an unfolding time of 600 s, P93A RNase A behaves the same as wild-type RNase A; however, at short unfolding times, there is a significant difference from wild-type RNase A. First, as seen in the single-jump folding experiments, there is no fast-folding species, but there is a very fast-folding species, U_{vf}, having a folding time constant of 150 ms. However, this U_{vf} species disappears much more rapidly than the U_{vf} species in wild-type RNase A. Also, the rate of formation of the U_m species is much faster in P93A than in wild-type RNase A as seen in Figures 7 and 8. The data were fit to the kinetic model shown in eq 3.



(3)

The designations of c and t and of the unfolded species and the rate constants are the same as in eq 2; however, the additional rate constant k^7 (instead of k^3) describes the cis to trans rate of the Tyr92−Ala93 peptide bond. This reaction was considered to be irreversible because such an isomerization for a non-proline peptide bond in an unfolded protein is unlikely to occur (see Discussion). Part of eq 2 is omitted from eq 3 because it is unlikely that a peptide bond preceding Ala93 adopts the cis conformation in the unfolded state.

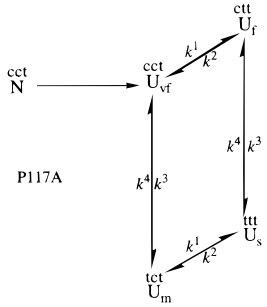
P114A RNase A shows the simplest kinetics for refolding in the double-jump experiments (Figure 9). A single slow-folding species, U_s, and the U_m and U_f species are observed after unfolding for 600 s. The first unfolded species to appear after unfolding for 0.2 s is the fast-folding species, U_f. There is no U_{vf} species in P114A RNase A. The data were fit to the kinetic model in eq 4.



(4)

Part of eq 2 is omitted from eq 4 for a similar reason, viz., that it is unlikely that a peptide bond preceding Ala114 adopts the cis conformation in the unfolded state.

The double-jump refolding kinetics of the P117A mutant of RNase A (Figure 10) at 1.3 M Gdn.HCl are essentially identical to those of wild-type RNase A. This mutant has a U_{vf} species with a folding time constant of 61 ms as well as U_f, U_m, and U_s unfolded species. The only difference seen in the single-jump folding experiments was a single slow-folding species, U_s, for P117A RNase A at all Gdn.HCl. concentrations. At 1.3 M Gdn.HCl, both wild-type and P117A RNase A exhibit a single U_s species. The data were fit to the kinetic model in eq 5.



(5)

Part of eq 2 is omitted from eq 5 for the same reason as in eqs 3 and 4, viz., Ala117 is unlikely to adopt a cis conformation in the unfolded state.

The rate constants, k^1-k^7, in eqs 2−5 describe the same process and thus should have the same values in each of the double-jump folding experiments. Therefore, the rate constants were varied to find values that fit the data from all four double-jump folding experiments simultaneously. These values are listed in Table 7.

DISCUSSION

The goal of this study is to identify regions in wild-type RNase A that are important in the initial steps of formation of three-dimensional structure. In order to determine which CFIS's of RNase A are affected by proline isomerization, it is necessary to obtain a model describing which X−proline peptide bond isomerizations are responsible for the unfolded species of RNase A. The data from the folding kinetics of each of the four proline mutants greatly aid in development of a model describing the refolding kinetics. By examining the differences in the distribution of unfolded species between wild-type RNase A and the four single proline-to-alanine mutants of RNase A, we can evaluate which X−proline peptide bond isomerizations are associated with which unfolded species. We are then able to propose a model (eq 2) that describes the coupling of cis/trans isomerization of the four X−proline peptide bonds and the conformational changes in the whole polypeptide chain during the refolding of the chemically denatured, disulfide-intact wild-type RNase A. The conformational changes can be very rapid even though accompanied by proline isomerization; the rate of the latter is itself influenced by the conformational changes.

In this model, we assume that only X−proline peptide bond isomerization is the cause of the different conformationally distinct unfolded species and that the folding pathways of the proline mutants are similar to those of wild-type RNase A. Equality of amplitudes of the various kinetic

Table 7: Values of the Rate Constants for the Kinetic Models (eqs 2−5) for Formation of the Unfolded Species of RNase A Described in the Text[a,b]

rate constant (s^{-1}) ($\times 10^3$)	k^1	k^2	k^3	k^4	k^5	k^6	k^7
	20.0	2.6	7.7	2.0	15.0	4.9	702

[a] The unfolding buffer, where the isomerizations occur, was 3.9 M Gdn.HCl at pH 2, at 15 °C. [b] These are rate constants for proline isomerization in an *unfolded* polypeptide chain. They differ from the rate constants (expressed as time constants) in Tables 2−5 because the latter refer to a combination of conformational (folding) changes *plus* proline isomerization.

phases at the six different final Gdn.HCl concentrations suggests that each kinetic phase represents a folding species; however, this is not necessarily true (Hagerman, 1977). Therefore, another assumption in this model is that each kinetic phase reflects a specific species. We also assume that the absorbance change upon folding, due to the burial of tyrosine residues in the folded protein, is a good probe for monitoring the relative amounts of native and unfolded proteins. In addition to the results presented in this paper, we also took account of results obtained by Houry et al. (manuscript in preparation), such as the characterization of the newly reported unfolded species, U_m, in developing this model.

Proline 42-to-Alanine 42 Mutant. The refolding of chemically denatured, disulfide-intact P42A RNase A has been described in detail previously (Dodge et al., 1994). It was shown that the number and relative amounts of the different conformationally distinct unfolded species of P42A RNase A were the same as those of wild-type RNase A in the single-jump folding experiments. The same folding kinetics are observed for P42A RNase A in this work; hence, it can be stated that the cis or trans isomeric state of the Lys41−Pro42 peptide bond makes no significant difference in the distribution of the unfolded species in chemically denatured, disulfide-intact RNase A.

Proline 93-to-Alanine 93 Mutant. Proline 93 is conserved in all of the sequenced homologous RNase A's (Beintema et al., 1986). For this reason, it would be suspected that the type VI turn made by the cis peptide bond at Tyr92−Pro93 is important for the native structure of RNase A. The results from the double-jump folding experiments in this work strongly suggest that, even when proline 93 is replaced by an alanine, a cis peptide bond in this turn between Tyr92 and Ala93 is formed. The first step in the double-jump folding experiment is the unfolding of the native protein. Immediately after the unfolding, peptide bonds that exist in multiple conformations (cis or trans) in the unfolded state will still be in the conformational state in which they were when in the native state. Then, after some time, an equilibrium between the cis and trans states of the peptide bond will be established. The time for the equilibrium to be established will depend on the rate of conversion between the cis and trans isomers of the peptide bond.

The rate constant for conversion from U_{vf} to unfolded species with non-native isomeric states in P93A RNase A, 0.702 s^{-1} (k^7 in Table 7), is much larger than the rates for conversion of U_{vf} to unfolded species with non-native isomeric states in wild-type RNase A (rates k^1, k^3, and k^5 in Table 7). The only difference between wild-type RNase A and P93A RNase A is the alanine residue at position 93. Thus, this increase in the rate constant for conversion of the U_{vf} species to other unfolded species must be due to this change.

In wild-type RNase A, 100% of the Tyr92−Pro93 peptide bonds in U_{vf} are in the cis conformation before further isomerization to the other unfolded species can take place (Houry et al., 1994). In the completely unfolded state, the Tyr92−Pro93 peptide bond is a mixture of cis and trans conformations (Adler & Scheraga, 1990). In the unfolded state of P93A RNase A, nearly 100% of the Tyr92−Ala93 peptide bond would be expected to be in the trans isomeric state because of the unfavorable energy of a non-proline cis peptide bond (Zimmermann & Scheraga, 1976). Again, the increase in the rate constant for the conversion of U_{vf} to other unfolded species in P93A RNase A must be due to the replacement of proline 93 by alanine 93; if the Tyr92−Ala93 peptide bond were trans in the native state, there would be no isomerization of this bond upon unfolding (a cis Tyr92−Ala93 peptide bond is energetically very unfavorable). Isomerization of other peptide bonds would be expected to occur at rates similar to those rates in wild-type RNase A. If, however, the Tyr92−Ala93 peptide bond was 100% cis in the native state, upon unfolding, this peptide bond would be energetically very unfavorable, and as Odefey et al. (1995) have shown, the cis-to-trans isomerization of non-prolyl peptide bonds has an increased rate over the cis-to-trans isomerization of prolyl peptide bonds. This is exactly what we observe in the double-jump folding experiment with P93A RNase A, i.e., rapid isomerization of the U_{vf} species to the U_m species.

In addition, mutants of RNase T_1 and carbonic anhydrase II variant have been shown to retain cis peptide bonds when a proline residue was changed to an alanine residue. With an NMR structure determination, Mayr et al. (1994) have shown the retention of the cis peptide bond in the P39A mutant of RNase T_1, and with a crystal structure determination, Tweedy et al. (1993) have shown the retention of the cis peptide bond in the P202A mutant of carbonic anhydrase II variant. Therefore, on the basis of the kinetics observed for the P93A mutant of RNase A and the fact that other proteins retain cis peptide bonds upon substitution of alanine for a proline residue, we conclude that the Tyr92−Ala93 peptide bond is in the cis conformation in native P93A RNase A. Presumably, as-yet-unidentified long-range interactions force the Tyr92−Pro93 and Tyr92−Ala93 peptide bonds to adopt the cis conformation.

Equation 3 indicates the proposed isomeric states for the peptide bonds preceding either proline or the alanine that replaces proline for the various unfolded species of P93A RNase A. The native wild-type RNase A structure is known, and for reasons stated above, P93A RNase A is thought to have the same conformation. The second unfolded species appearing immediately from the U_{vf} species is the U_m species. The expected rapid isomerization of the cis Tyr92−Ala93 peptide bond results in the U_m species which has all native peptide bond conformations except for a non-native trans peptide bond between Tyr92 and Ala93. This rapid isomerization is expected to be essentially irreversible in the unfolded protein, and the rate constant describing this isomerization, k^7, is unique to P93A RNase A. There is no U_f species in P93A, and the remaining slow-folding species must be a mixture of the remaining possible isomeric states.

Proline 114-to-Alanine 114 Mutant. The single-jump folding kinetics for P114A differ from those of wild-type only in the distribution of the U_s^{II} and U_s^{I} unfolded species at different Gdn.HCl folding conditions. The double-jump folding experiment shows that there is also no very fast-folding species, U_{vf}. The unfolded species that has all the native conformational isomeric states of the peptide bonds has a time constant and amplitude comparable to those of the U_f species of wild-type RNase A. This suggests that, in wild-type RNase A, the difference between the U_{vf} and U_f species is an incorrect isomer of the Asn113−Pro114 peptide bond [as suggested by Houry et al. (1994)]. Equation 4 indicates the proposed grouping of the U_m and U_s unfolded species in P114A RNase A. The difference in the relative amounts of slow species between P114A and wild-type RNase A observed in the single-jump folding experiments is due to the fact that the U_s species is a distribution of unfolded species, and eq 4 makes no distinction between the U_s^{I} and U_s^{II} folding species.

Proline 117-to-Alanine 117 Mutant. The single-jump folding experiments on P117A RNase A show only one slow-folding species at all final Gdn.HCl concentrations. This indicates that separation into two refolding phases of the U_s species in wild-type RNase A is due, in part, to a non-native isomer of the Val116−Pro117 peptide bond. The double-jump folding experiments with P117A show kinetics nearly identical to that of wild-type RNase A, and thus, the isomeric state of the Val116−Pro117 bond of wild-type RNase A must affect only the slow-folding species (U_s^{I} and U_s^{II}). In the double-jump folding experiments, U_s^{I} and U_s^{II} in wild-type RNase A are indistinguishable and hence are grouped together as U_s in eq 2; thus, P117A RNase A has the same observed refolding kinetics as wild-type RNase A at 1.3 M Gdn.HCl. However, since the data from the single-jump folding experiments indicate that the U_s species in P117A is a single species while U_s in wild-type RNase A is more than one species, the model for P117A (eq 5) differs from the model for wild-type RNase A (eq 2).

Wild-Type RNase A. On the basis of the information from the experimental results on the four proline mutants, it is possible to propose a model for the folding of the various unfolded species of wild-type RNase A. The proline mutants of RNase A have three-dimensional structure nearly the same as RNase A on the basis of the fact that their activity on cCMP is similar to that of wild-type RNase A. The folding rates and amplitudes of the proline mutants are also very similar to those of wild-type RNase A. Therefore, we suggest that the proline mutants follow the same folding pathway as wild-type RNase A with the only difference in the pathway arising from effects created by the replacement of a single proline with alanine. The very fast-folding species in wild-type RNase A has the same distribution of peptide bond isomers as that of native RNase A (Houry et al., 1994). We propose that the fast-folding species (U_f) consists of two isomers. One has all the proline isomers in the native state except that the Asn113−Pro114 peptide bond is in a trans conformation and not a cis conformation as in the native state. The other has all isomers in the native state except that the Val116−Pro117 peptide bond is in the cis conformation. Equation 2 shows the U_f species consists of these two isomeric states, ctt and ccc. This proposal is based on the fact that the folding rate constant and relative amplitude of the fast-folding species of P114A RNase A are the same as those of the U_f species of wild-type RNase A and that the P114A mutant of RNase A has a trans Asn113−Ala114 peptide bond. In addition, P93A RNase A does not have an unfolded species with a rate constant similar to the U_f species, which is consistent with the model because P93A RNase A must have an additional trans state besides the one for Asn113−Pro114 [Tyr92−Ala93 is always trans, except upon immediate unfolding (i.e., in U_{vf}), in P93A RNase A].

We propose that the medium-folding species of wild-type RNase A consists of two unfolded species. The first has only the Tyr92−Pro93 peptide bond in the non-native (trans) state. The first unfolded species to appear after the isomerization from cis to trans of the Tyr92−Ala93 peptide bond in P93A RNase A is a species having a folding rate similar to the folding rate of the medium-folding species in wild-type RNase A. The second has only the Tyr92−Pro93 peptide bond in the native state since, in the double-jump experiments on P114A RNase A, we observe a U_m species and P114A does not have the Asn113−Pro114 peptide bond in the cis state.

The minor slow-folding species, U_s^{I}, is a species having a non-native (cis) Val116−Pro117 peptide bond. This is based on the fact that elimination of the possibility of a cis peptide bond between residues 116 and 117 in P117A RNase A results in the disappearance of the minor slow-folding species U_s^{I}. In the double-jump refolding experiments at 1.3 M Gdn.HCl, it is not possible to distinguish between the U_s^{II} and U_s^{I} species. Since the Lys41−Pro42 peptide bond state does not affect folding (Dodge et al., 1994), and all species with one non-native peptide bond have been assigned to other unfolded species, the remaining combinations must belong to the U_s species [provided no other type of isomerization besides X−Pro peptide bond isomerization occurs (Mui et al., 1985)]. Equation 2 shows the remaining species belonging to the U_s folding species.

Figures 7−10 show the results of the double-jump folding experiments. The values of k^1−k^7 in eqs 2−5 were varied to give the best fit to all the experimental points for wild-type and the four proline mutants of RNase A simultaneously. The rate constants thus obtained are listed in Table 7. Examination of the solid lines in Figures 7−10 shows that these values for the rate constants do not run exactly through every data point. However, given the inherent uncertainty in determining the relative amplitudes of the various folding phases, we believe that this model is consistent with our experimental results.

Comparison with Previous Models. Schultz et al. (1992) have also carried out experiments on several proline mutants of RNase A. They showed that the single mutants of the two cis proline residues in RNase A can be expressed and folded to native structures. Since their mutants have an N-terminal methionine residue, a direct comparison with our mutants without an N-terminal methionine residue is not possible. However, the thermal and Gdn.HCl stabilities of our mutants are consistent with the thermal and Gdn.HCl stabilities of their mutants.

The kinetic experiments of Schultz et al. (1992) used circular dichroism and fluorescence intensity to detect the slow-folding species of RNase A and the proline mutants. They stated that the refolding behavior of their P93A mutant of RNase A was unexpectedly complex and that they could not explain this behavior. However, in the abstract of their paper, they suggested that the observed complexity of the

P93A mutant of RNase A may possibly result from a cis Tyr92−Ala93 peptide bond, but they did not elaborate on this statement. In this work, we provide a model, eq 3, and a rate constant, k_7, for the proposed cis-to-trans isomerization of the Tyr92−Ala93 peptide bond. Schultz et al. (1992) used manual mixing techniques for their double-jump unfolding/folding experiments and, therefore, could not observe events occurring before 15 s. In our stopped-flow double-jump unfolding/folding experiments on P93A RNase A, we observe only the U_{vf} species, which we propose has a cis Tyr92−Ala93 peptide bond, at unfolding times less than 2 s.

Schultz et al. (1992) reported that the main conclusion from their work is that the two cis proline residues in RNase A are responsible for the major slow-folding species, U_s, of RNase A. The results of our experiments on P117A RNase A show that, in addition to the cis peptide bonds preceding proline 93 and proline 114, the trans peptide bond preceding proline 117 is also responsible for the slow-folding species, U_s, in RNase A.

Houry et al. (1994) have proposed a model for the distribution of peptide bond isomeric states in unfolded wild-type RNase A. With the additional information obtained in this work, the isomerizations responsible for the various unfolded species could be assigned, and the rate constants for the cis/trans isomerizations of the peptide bonds in unfolded RNase A could be determined.

CONCLUSIONS

By detailed kinetic studies of four proline mutants of ribonuclease A, a model indicating the isomeric states of the X−Pro peptide bonds in *unfolded* wild-type RNase A has been developed. This model shows that the isomeric state of the Lys41−Pro42 peptide bond is not rate-determining in the refolding of disulfide-intact, chemically denatured RNase A. Therefore, the region around proline 42 in RNase A is not a CFIS. On the basis of the model, the isomerization state of the Tyr92−Pro93, Asn113−Pro114, and Val116−Pro117 peptide bonds affects folding to the native state. This result supports the fact that the region around the type VI turn at Tyr92−Pro93 and the region encompassing residues 106−118 (Matheson & Scheraga, 1978; Montelione & Scheraga, 1989) are chain-folding initiation sites and that three-dimensional structure must form in these regions before the protein can fold to the native state.

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