Schellman, J. A. (1955) *C. R. Trav. Lab. Carlsberg, Ser. Chim. 29*, 230.
Scheraga, H. A. (1980) in *Protein Folding* (Jaenicke, R., Ed.) p 261, Elsevier, Amsterdam.
Scheraga, H. A. (1981) *Biopolymers 20*, 1877.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1.
Tanford, C., Kawahara, K., & Lapanje, S. (1966) *J. Biol. Chem. 241*, 1921.
Wang, M. C., & Uhlenbeck, G. E. (1945) *Rev. Mod. Phys. 17*, 323.
White, F. H., Jr., Bello, J., Harker, D., & Jarnette, E. De. (1961) *J. Biol. Chem. 236*, 1353.

Observation of Intermediates in the Folding of Ribonuclease A at Low Temperature Using Proton Nuclear Magnetic Resonance†

Roger G. Biringer and Anthony L. Fink*

ABSTRACT: The refolding of ribonuclease A (RNase A) has been investigated in aqueous methanol cryosolvents in the 0 to −20 °C range. When a thermally unfolded sample was brought under renaturing conditions (e.g., −16 °C, 35% methanol, pH* 2.8), the refolding, as monitored by the absorbance change at 286 nm (which reflects the degree of solvent exposure of Tyr), was triphasic and took approximately 1 h for completion. The 360-MHz proton nuclear magnetic resonance (NMR) spectrum of the native enzyme in either 35% or 50% aqueous methanol is very similar to that in aqueous solution. When the refolding of RNase A was monitored in the subzero temperature range with the signals of the His C2 protons, new resonances rapidly appeared, in addition to those from native protein. The new resonances are attributed to a partially folded intermediate state that has a relatively compact structure. Time-dependent changes were observed in the areas of the resonances from both native and partialy folded species. The rates of peak area reduction for the intermediate state were the same as those for the increase in area of the native resonances, and similar to those for the second phase observed in the absorbance experiments. The results are consistent with the slow-refolding form of RNase A consisting of a least two distinct populations. A model for the folding of RNase A is proposed.

Recent evidence suggests that the folding of small proteins such as RNase A[1] is intermediate controlled (Cook et al., 1979; Schmid, 1981; Schmid & Baldwin, 1979; Blum et al., 1978); that is, it is determined by the structure and stability of partially folded intermediate states (Ptitsyn & Rashin, 1975). The present study was undertaken to shed light on the nature of such intermediate species.

The refolding of RNase A has been extensively studied, especially by Baldwin and co-workers [e.g., Baldwin (1975, 1980) and Baldwin & Creighton (1980)], under a variety of conditions in aqueous solutions. Evidence for at least two different intermediates has been reported. On the basis of competition experiments between amide proton exchange and folding, Schmid & Baldwin (1979) demonstrated the presence of an early intermediate, lacking significant compact, globular structure. An extensive series of experiments by Cook et al. (1979) and Schmid (1981; Schmid & Blaschek, 1981) has shown that a nativelike intermediate, I_N, is formed during folding under "strongly" native conditions. Pro-93 has been proposed to be in the nonnative trans configuration in I_N. This intermediate can bind specific inhibitors, is catalytically active, and is presumed to have a structure virtually identical with that of the native protein except in the immediate vicinity of the loop around Pro-93.

The proton NMR spectrum of a protein contains a large amount of potential structural information. If partially folded intermediates are present during refolding, new transient resonances may be observed whose chemical shifts are different from those of the unfolded and native states. In the present study we have focused on the four His C2 protons since their resonances are well resolved and separated from those of other protons. There is one previous report in which NMR has been used to monitor the kinetics of the refolding of RNase A (Blum et al., 1978). In this instance RNase A was heated to 46 °C, pH 2.1, and rapidly quenched to 10 °C, and spectra were collected at 1-min intervals. A new resonance, in addition to those from unfolded and native states, was detected and attributed to the C2 proton of His-12 in an environment similar to that in the S peptide. It was concluded that formation of the N-terminal helix (residues 3–13) occurred at an early stage in refolding, prior to the formation of significant structure about the remaining three His residues.

We have shown that *methanol-based cryosolvents at subzero temperatures have no adverse effects on the catalytic and structural properties of RNase A* (Fink & Grey, 1978; R. G. Biringer and A. L. Fink, unpublished results; A. L. Fink, D. Kar, and R. Kotin, unpublished results). Experiments at subzero temperatures have been shown to be valuable in the detection of normally transient intermediates in enzyme catalysis (Fink, 1977; Douzou, 1977; Fink & Cartwright, 1981). Preliminary experiments have indicated that such an approach might also be very useful in protein folding studies (Fink & Grey, 1978).

We have recently used high-resolution proton NMR to monitor the unfolding of RNase A in the presence of methanol (R. G. Biringer and A. L. Fink, unpublished results). The investigation revealed that partially folded intermediates, in

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[1] Abbreviations: RNase, ribonuclease; NMR, nuclear magnetic resonance; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; N, native; U, unfolded; δ, chemical shift; S/N, signal to noise ratio; Gdn·HCl, guanidine hydrochloride; 2′,3′-cCMP, cytidine cyclic 2′,3′-phosphate; 2′-CMP, cytidine 2′-phosphate.

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slow exchange with both native and unfolded protein, as well as intermediates in fast exchange with each other and with native enzyme, could be detected. The data were consistent with several nativelike (compact) and several partially folded (less compact) species being present during the transition from N to U. The presence of methanol greatly decreased the cooperativity of the transition, and therefore must stabilize the partially folded states.

The folding of RNase A is complicated by the isomerization of proline residues. In the unfolded state two configurations exist, U_F, which has the four Pro residues in the same cis/trans configurations as those found in the native protein and which refolds very rapidly, and U_S, in which Pro-93 and Pro-114 have the nonnative trans configuration and which refolds relatively slowly (Baldwin, 1975; Brandts et al., 1975; Cook et al., 1979; Schmid & Blaschek, 1981; Henkens et al., 1980).

In the present study we have monitored the NMR signals from the four His C2 protons during refolding under native conditions, at subzero temperatures, in order to detect the presence of intermediates on the folding pathway. Kinetic comparisons have also been made with refolding rates determined from absorbance changes due to Tyr burial.

Materials and Methods

Methanol-d_4 was obtained from Stohler Isotope Chemicals Inc. and D_2O from Bio-Rad Laboratories. Ribonuclease A was purchased from Calbiochem-Behring Corp. The enzyme was further purified on a Sephadex SPC-25 colun (0.13 M phosphate, pH 6.5). The sample was ultrafiltered 4–5 times with a 50-fold excess of distilled water to remove residual phosphate in an Amicon ultrafiltration assembly with a PM-10 membrane and then lyophilized. Phosphate analysis revealed that the resulting RNase A samples contained no more than 0.24 mol % phosphate.

The peptide backbone protons of RNase A were exchanged with D_2O by Markley's (1975) procedure E. By repetition of the process 2 or 3 times,, the residual HOD peak could be made very small. The product was lyophilized and stored at −20 °C. Selective deuteration was accomplished as follows: Purified RNase A was dissolved in D_2O (99.8%) (40 mg/mL) and the pH* adjusted to 8.00 with NaOD. The solution was then incubated at 40 °C. After 14 days the sample was allowed to cool to ambient temperature, and the pH* was adjusted to 3.00 to quench the exchange reaction. A white precipitate formed during the process and was removed by centrifugation. The solution was then lyophilized. The spectrum of the selectively deuterated protein in 35% methanol-d_4, at 25 °C, was identical with that of native RNase A except for His C2 peak areas. This observation, particularly the absence of extraneous peaks, indicates the homogeneity of the selectively deuterated enzyme.

Solutions of deuterium-exchanged ribonuclease A in 35% and 50% methanol-d_4 were prepared as follows: The cryosolvents were mixed on a v/v basis, i.e., 35:65 methanol-d_4:D_2O (99.9%), and mixed with lyophilized enzyme. The pH* was then adjusted with DCl or NaOD as necessary. DSS was generally added to the solvent prior to mixing with RNase. The pH* values reported herein are the observed pH meter readings obtained at 25 °C with a glass combination electrode. The values of pH* thus represent the apparent protonic activity in the deuteriocryosolvent (Hui Bon Hoa & Douzou, 1973).

Absorbance measurements of the folding were performed at 286 nm on a Cary 118 spectrophotometer. Typical concentrations of RNase A were 15 μM. An insulated, thermostatted brass-block holder was used to maintain constant temperature in the sample cuvette. The coolant used was ethanol from a Neslab RTE-8 bath. In a typical refolding experiment 20 μL of RNase A (0.75 mM, pH* 3.0, in 35% or 50% aqueous methanol) was taken up in a microsyringe and incubated at 70 °C for 10 min. The syringe contents were then injected into the thermostatted cuvette containing 1.0 mL of 35% or 50% methanol cryosolvent at the desired temperature. After the contents were mixed, the absorbance changes were followed. Protein stock solutions were made up immediately before use.

NMR Measurements. All measurements were performed with a modified Bruker 360-MHz spectrometer, equipped with a Nicolet 1180 data system, unless otherwise noted. All chemical shifts are referred to an internal DSS standard. At each temperature the spectrum was calibrated by setting the reflected DSS peak to 5000 Hz.[2] Peak areas were integrated by electronic integration.

All NMR spectra were obtained with a Redfield 21412 pulse sequence in which the shortest pulse length was 71 μs. The temperature was calibrated with an external methanol standard. The time required for temperature equilibration in refolding experiments was determined as follows: An aliquot of the sample solution was placed in an NMR tube and allowed to equilibrate at the desired subzero temperature. The dispersion signal used for locking the sample was centered on the oscilloscope. The spectrometer was then tuned to this sample, which was then replaced by the quenched refolding sample. The dispersion signal returned to the center of the oscilloscope within 3 min, indicating that temperature equilibration of the refolding solution had been achieved within this time. Spectra were collected at 1-min intervals, with 60 scans per spectrum.

Kinetic Analysis. The kinetics of refolding were analyzed by a linear expansion least-squares algorithm described by Bevington (1969). The analysis routine is a modification of one described by Morris et al. (1980), which has been recompiled to run on a floppy-disc-based microcomputer system, running under the CP/M operating system. Kinetic analyses were also carried out graphically, by means of a least mean squares fit procedure.

Results

Native Conditions in Aqueous Methanol. In order to carry out folding experiments under native conditions at subzero temperatures, it was necessary to determine the appropriate experimental conditions. We have established these as follows. The melting curves for RNase A in 35% and 50% methanol at pH* 2.8, as determined from changes in absorbance at 286 nm, are shown in Figure 1. In both cases the transition begins at or above −10 °C. Similar curves have been obtained from measurements of NMR peak areas (R. G. Biringer and A. L. Fink, unpublished results). These show the beginning of the thermal denaturation transition to be −10 °C for 50% methanol and +10 °C for 35%. This was confirmed in the following experiments. These melting curves show considerable deviation from that expected in a two-state system. This lack of cooperativity is attributed to the population of partially folded states during unfolding in the presence of methanol.

The ^1H NMR spectrum of RNase A in 35% methanol, pH* 2.8, at −16 °C is shown in Figure 2A. A similar spectrum is also observed with 50% methanol under these conditions. These spectra closely resemble those of the native protein in aqueous solution. No evidence for any nonnative resonances is observed. In addition the Arrhenius plot for the catalytic reaction with 2′3′-cCMP in 50% methanol shows no deviation

[2] The resulting chemical shifts are identical with those obtained in a subsequent one-pulse experiment.

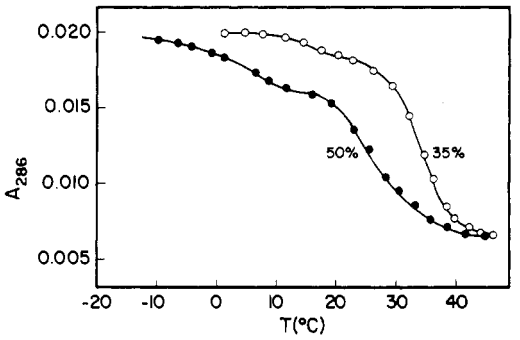

FIGURE 1: Thermal denaturation transition curves for RNase A in 35% and 50% methanol, pH* 2.8. The transition was measured by changes in the absorbance at 286 nm, which measures the solvent exposure to tyrosine residues.

from linearity below −10 °C (unpublished data).

In Figure 2B,C we have shown the spectra of RNase A in 35% and 50% methanol, pH* 3.0, at 69 °C, the conditions that were used to unfold the protein (see below). Even at this temperature there is evidence for the presence of some nonrandom structure, as witnessed by the His C2 doublet for the spectrum from 35% methanol, the broad, asymmetric His C2 resonance for 50% methanol, and the multiplet shown for the tyrosines, instead of two doublets. The high rate of solvent evaporation prevents use of temperatures much higher than 70 °C in these experiments. Studies in aqueous solution have shown that under apparently fully unfolded conditions the NMR spectrum of the His C2 signal also reveals similar traces of structure [e.g., Blum et al. (1978) and Howarth & Lian (1981)].

We have previously shown that increasing concentrations of methanol stabilize partially folded intermediates in the *unfolding* of RNase A (R. G. Biringer and A. L. Fink, unpublished results). A representative NMR spectrum of such partially folded states is given in Figure 2D for purposes of comparison.

Refolding Monitored by Absorbance Changes. In order to determine the appropriate choice of NMR parameters, we investigated the refolding reaction by using absorption spectrophotometry. RNase A has no tryptophan residues, but has six tyrosines, four of which are buried or partially buried in the native structure (Tyr-25, -73, -92, and -97). The degree of solvent exposure of these Tyr residues may be determined from changes in A_{286}. The time-dependent changes in A_{286} observed when RNase A was denatured at 70 °C, pH* 3.0, then injected into 35% methanol (65% D_2O) at −16 °C, are shown in Figure 3A,C. The data were best fit by a model of three consecutive first-order reactions, with rate constants (with standard deviations in parentheses) of 2.8 (±0.05) × 10^{-2} s^{-1}, 4.5 (±1.0) × 10^{-3} s^{-1}, and 3.3 (±1.4) × 10^{-4} s^{-1}, respectively. The triphasic nature of the reaction is quite evident in semilog plots of the data, shown in Figure 3B,D. These results suggested that it should be possible to acquire NMR spectra during the time period of the second and third phases of the reaction.

Refolding Monitored by NMR. A number of technical problems had to be overcome in order to obtain useful NMR

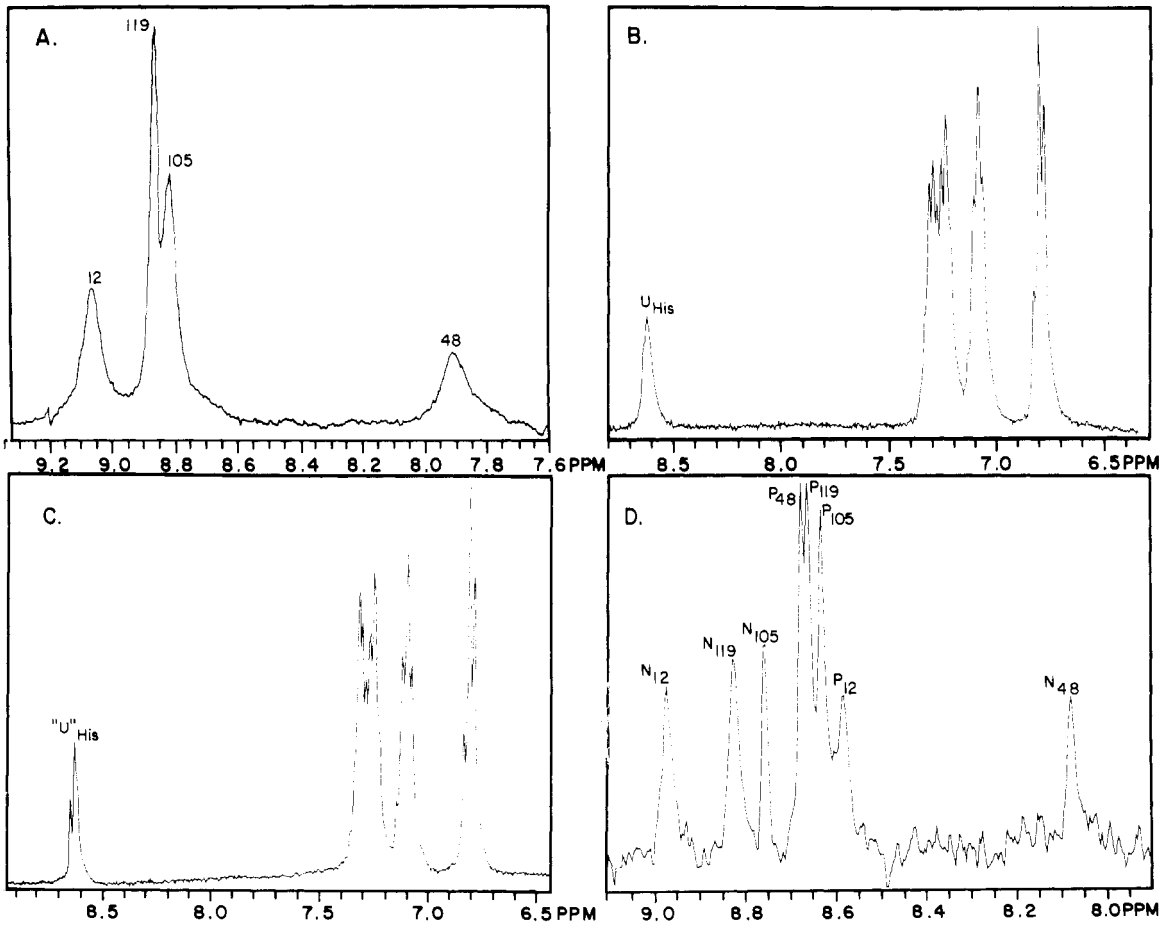

FIGURE 2: Proton NMR spectra of RNase A: (A) His C2 region in 35% methanol-d_4, pH* 2.8, −16 °C; (B) aromatic region (His, Tyr, and Phe) of RNase A in 35% methanol-d_4, pH* 2.8, 69 °C; (C) aromatic region in 50% methanol-d_4, pH* 2.8, 69 °C; (D) His C2 region of RNase A in 50% methanol-d_4, pH* 3.0, 33.1 °C, showing the resonances of partially folded intermediate states in slow exchange with native protein. Note the different ranges of chemical shift scales.

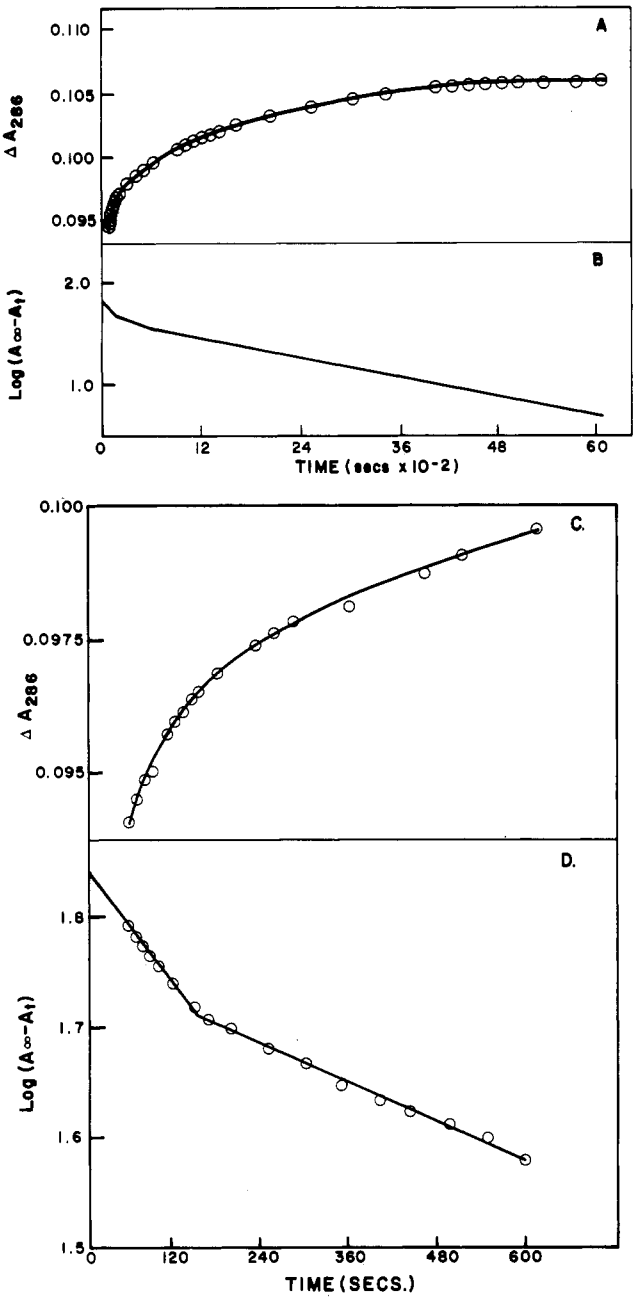

FIGURE 3: Refolding of RNase A in 35% methanol, pH* 2.8, −16.1 °C, as monitored by the change in absorbance at 286 nm: (A) time-dependent change in A_{286}; (B) semilog plot of data of (A) to show triphasic nature of the reaction; (C) and (D) expansion of data of (A) and (B), respectively.

spectra during refolding. The major problems were associated with rapidly changing the sample conditions from strongly denaturing to strongly native and with obtaining a sufficient S/N, given the time constraints imposed by the protein solubility and the kinetics of refolding. For practical reasons, relating especially to RNase A solubility, temperatures above −20 °C were deemed most appropriate for these experiments.

Several different procedures were investigated for rapidly changing the sample from denatured to native conditions. The two best methods, which gave similar results, were (a) heat denaturation of the protein in a syringe (70 °C, 10 min) and injection of the contents into the NMR tube positioned in liquid nitrogen (the frozen sample was then placed in the NMR probe at the desired temperature) and (b) heat denaturation of the sample in the NMR tube, followed by quenching of the tube in liquid nitrogen to a few degrees below

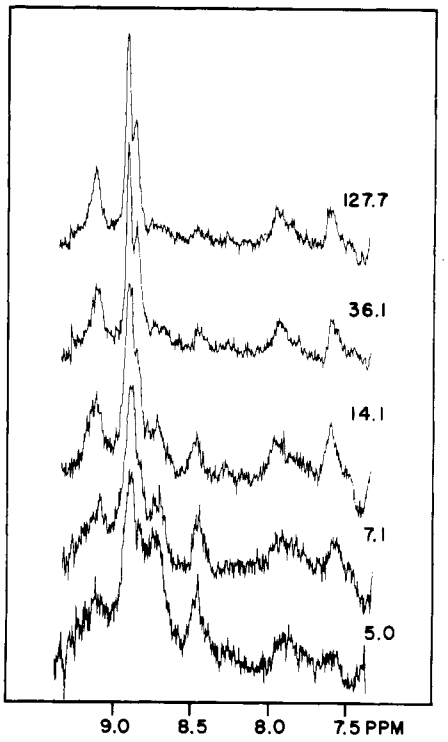

FIGURE 4: His C2 region of RNase A during refolding in 35% methanol-d_4, pH* 2.8, −16 °C. Spectra were collected at 1-min intervals. The times (in minutes) corresponding to the resonances in the figure are shown to the right of the spectra.

the desired temperature, prior to placing it in the NMR probe. Control experiments indicated that the sample reached −20 °C within 6 s. Quenching techniques involving dilution of concentrated protein samples (e.g., to allow unfolding by high guanidinium chloride concentrations) were found unsatisfactory due to limited solubility of the enzyme and to insufficient final sample concentrations.

Refolding experiments were carried out at several temperatures in the 0 to −20 °C range, with both 35% and 50% methanol solvent systems. In general the data are quite similar (although of course the rates are different at different temperatures). Consequently only one particular data set, namely, 35% methanol, pH* 2.80, −16 °C, will be considered here. Both quenching techniques were used in these experiments, and similar results were obtained from either technique.

During refolding spectral acquisitions were collected in blocks of 60, giving spectra at 60-s intervals. This was the minimum time interval in which a resolved spectrum could be accumulated under the experimental conditions. Some improvement in S/N could be obtained by adding two or three such spectra together; however, the rapidly changing peak areas severely limit such signal averaging. Some representative spectra are shown in Figures 4 and 5. Due to the short aquisition periods and fast relaxation caused by reduced mobility at −16 °C, the S/N is rather poor.

Spectra from the earliest time periods in refolding showed new resonances not observed from the native or unfolded protein. The chemical shift of the His C2 signal of unfolded RNase A is expected at 8.63 ppm (see Discussion). Even in the earliest spectra obtained (3 min) there is no evidence of any significant concentration of unfolded protein. The new resonances are attributed to His residues in a partially folded state for reasons to be elaborated subsequently and are labeled P_1, P_2, P_3, P_4, and P_5 (Figure 5). Resonance P_5 is readily resolved in selectively deuterated samples, and at late times in nonselectively dueterated samples (Figure 6). Examination

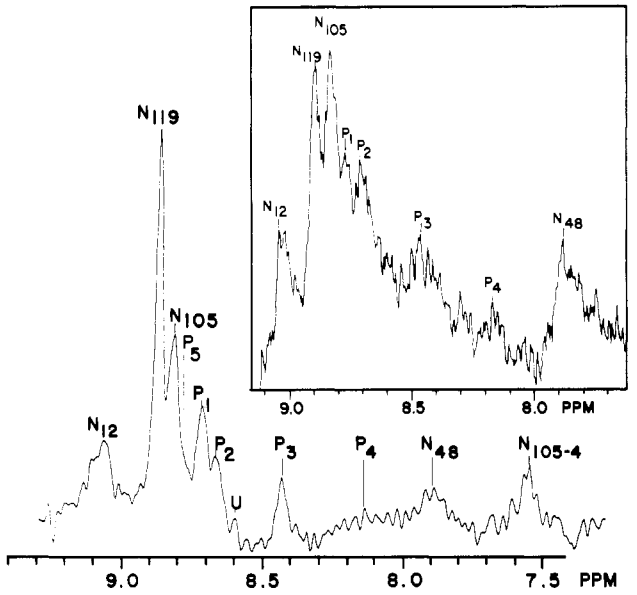

FIGURE 5: Spectra of His C2 region during refolding in methanol-d_4, pH* 2.8, −16 °C: summed spectra from 5.1 to 10.1 min in 35% methanol; (inset) spectrum at 3.4 min during the refolding of RNase A in 50% methanol, pH* 3.26, −10.1 °C. The lower spectrum has been resolution enhanced by double exponential multiplication.

Table I: Rate Constants for Change in Peak Areas Observed in Refolding RNase A in 35% Methanol, pH* 2.8, −16.1 °C

NMR peak[a]	$k_{obsd} \times 10^3$[b] (s^{-1})	NMR peak[a]	$k_{obsd} \times 10^3$[b] (s^{-1})
His-12	2.1	$P_1 + P_2$	2.3 ± 0.5
His-48	2.6	P_3	1.0
His-105	2.4	ΣN[c]	2.5 ± 0.8
His-119	4.1	ΣP[c]	2.6 ± 0.9

[a] His-x refers to the native resonance. [b] Typical standard deviations were ±25%. [c] Summed peak areas for the native or partially folded resonances.

of the data from Figure 4, and from similar experiments not shown, indicates that the chemical shifts of all the resonances are constant with time, whereas the peak areas show time-dependent changes.

The times for the spectra shown in Figures 4 and 5 are accounted for as follows: zero corresponds to the time of injection of the denatured sample into the cold NMR tube. Because of temperature equilibration the first useful spectra are obtained in 3–4 min (Figure 5B).

The peak areas of the resonances assigned to partially folded species, which are in slow exchange with the native material, decrease as refolding progresses, whereas the areas of the native peaks increase. Ultimately the spectrum becomes essentially identical with that of the native protein under similar experimental conditions (compare top spectrum of Figure 4 to that of Figure 2A).

Kinetics of Refolding. The time-dependent changes in peak area are shown in Figure 7 for some of the resonances corresponding to the native and partially folded species. The calculated rate constants are given in Table I. The areas of the individual His C2 resonances have been calculated as a percentage of the total area corresponding to His C2 peaks. Areas for infinite time were assumed to be equivalent to those from the same sample (at −16 °C) in a spectrum of the native protein taken prior to unfolding. As can be seen in Figure 7, the total area of native material at long time periods corresponds to only 90% of the expected total. There are two reasons for this. First, the low S/N means that considerable

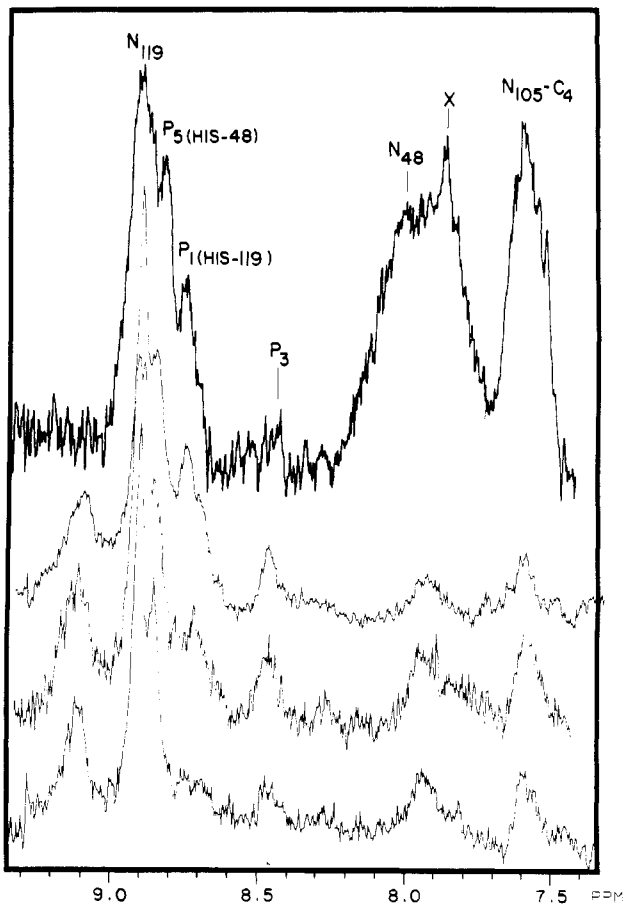

FIGURE 6: Spectra of His C2 region during refolding in 35% methanol-d_4, pH* 2.8, −16 °C. The upper spectrum is that of a partially deuterated sample (see text), most of the signal coming from His-48 and His-105. The spectrum was taken at 7.39 min. The lower spectra are ones from the refolding of nonselectively deuterated enzyme, taken under similar conditions. The times associated with each of these spectra are, from top to bottom, 7.63, 14.1, and 36.2 min. The peak labeled X is believed to be a quadrature glitch and was not present in a spectrum of the sample taken in D_2O.

error is present in the peak areas of the partially folded species at longer time periods. We estimate that this error could account for as much as 4–5% in the summed areas of resonances from the partially folded material. Second, it is possible that some of the unfolded protein may not refold, perhaps due to aggregation.[3]

The similarity in chemical shift for P_1 and P_2 made it impossible to accurately resolve the individual peak areas; consequently, we have used the sum of their areas. Similarly the area for P_5 could not be obtained due to severe overlap with that of His-105 (native).

Analysis of the peak area data indicates that 52 ± 5% of the total was completely refolded within 3 min, 37% refolded with a rate constant of 2.6×10^{-3} s^{-1}, and 11% either did not refold or is lost in the poor S/N. The significant points to note are that (1) the rate of loss in peak area for the partially folded species equals the rate of increase in peak area for the resonances from the native state (Figure 7A) and (2) the rate constant from the NMR experiments [$(2.6 \pm 0.9) \times 10^{-3}$ s^{-1}] is similar to the second phase observed in the refolding reaction monitored by changes in absorbance at 286 nm [$(4.5 \pm 1.0) \times 10^{-3}$ s^{-1}].

[3] Certain observations indicate that partially folded intermediate states and unfolded protein may be less soluble than the native state and, hence, more prone to aggregation at high protein concentrations.

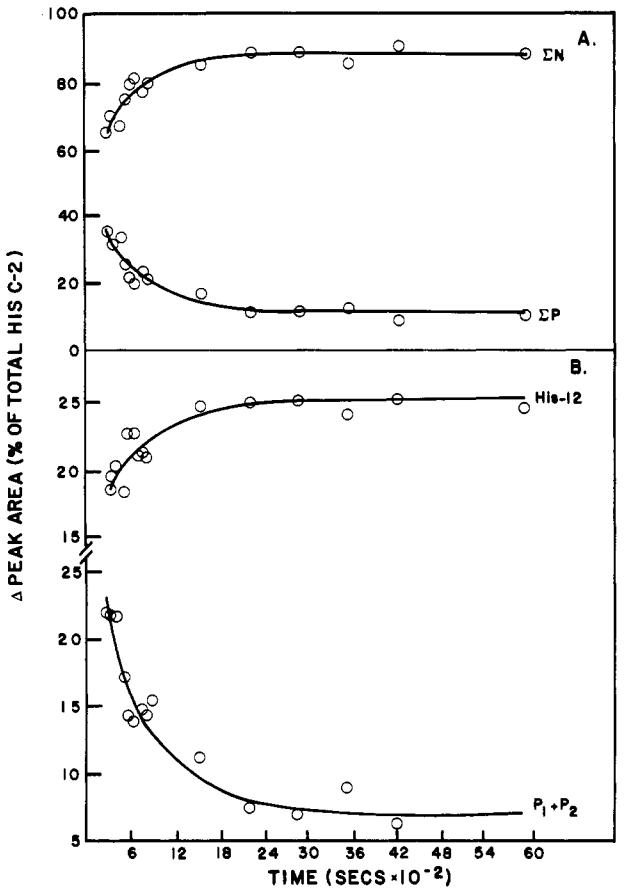

FIGURE 7: Time-dependent changes in resonance areas during refolding in 35% methanol, pH* 2.8, −16 °C: (A) changes in summed areas of all resonances from native state (top) and in summed areas of resonances from partially folded species (bottom); (B) area changes in resonance from His-12 in the native state (top) and from resonances P_1 and P_2 from the partially folded state (bottom). The circles are experimental data, and the solid curves are calculated for a monoexponential reaction with the rate constants given in Table I.

Extrapolation to time zero of the NMR reaction in which the partially folded species converts into the native material indicates that 52 ± 5% of the total RNase A present was already in the native state. We ascribe the formation of this native material to four possible sources. (1) One is fast-refolding protein (U_F) (with the correct proline configurations, see Discussion), which would be expected to fold sufficiently rapidly to have all converted into native enzyme within the first 3 min of the reaction. (2) Although the quenching of the denatured sample was very rapid, some may have renatured during this process, although we would not expect more than a few percent. (3) Some of the partially folded intermediates formed late in folding may have an NMR spectrum very similar to that of the native state and, thus, with the present resolution, are indistinguishable from native. (4) Some of the slow-refolding species may have refolded to nativelike material within the first 3–4 min of the refolding reaction.

Analysis of the data for the refolding monitored by absorbance change indicates that at the beginning of phase 2, the reaction corresponding to that observed by NMR, 56 ± 6% of the signal corresponds to that of native enzyme. [The total absorbance change expected was calculated from the total change observed in thermal denaturation (melting) curves.]

The kinetics of 2′-CMP binding during refolding were observed spectrophotometrically at 242 nm. The data indicate that under the conditions of the NMR refolding experiment 100% of the binding activity is recovered at the completion of the reaction in which partially folded material converted into native material (20 min).

Discussion

Questions of protein folding mechanisms have focused on random, nucleation-controlled and intermediate-controlled pathways. Previous studies strongly suggest that, at least in the case of RNase A, an intermediate-controlled mechanism prevails. That is to say the relative stabilities of partially folded intermediate states determine the kinetics and pathway of folding. The results of the present study confirm such a model. Of particular interest is the nature of the folding intermediates. An obvious question that arises concerning this investigation is its relevance to folding under physiological conditions. Undoubtedly there will be differences in the folding pathway when cosolvent is present. However, it seems reasonable, given that the protein refolds to essentially the same native state as that in aqueous solution, and with similar overall kinetics, to consider that at the very least, the reaction in aqueous methanol serves as a model for that in aqueous solution.

Fast- and Slow-Refolding Species. Baldwin and co-workers (e.g., Baldwin, 1975; Hagerman & Baldwin, 1976; Nall et al., 1978; Hagerman et al., 1979) have shown that unfolded RNase A exists as an equilibrium mixture between two configurationally different forms, U_S and U_F, in which U_F refolds rapidly whereas U_S refolds slowly. Considerable evidence supports the suggestion of Brandts (Brandts et al., 1975) that U_F and U_S differ in their configuration about one or more proline residues. In particular it appears that Pro-93 is probably in the nonnative trans configuration in U_S (Schmid, 1981; Schmid & Blaschek, 1981). It is also probable that more than one form of U_S exist, differing in the number of incorrect Pro isomers (Schmid & Blaschek, 1981).

Under the typical denaturing conditions used in aqueous solvent studies (e.g., 5 M Gdn·HCl, pH 2, 40 °C) the ratio of U_F to U_S is 1:4, i.e., 80% slow refolding. This ratio has been shown to be pH independent above pH 2 (Hagerman & Baldwin, 1976). In the present study the unfolding conditions are rather different, namely, 35% or 50% methanol, pH* 3.0, 70 °C. The amplitude for the three phases observed by monitoring refolding by absorbance changes accounts for 70 ± 3% of the expected change; i.e., slow-refolding species correspond to 70% of the total. The difference between this value and that of 80% reported for the denaturation in aqueous solution is probably within experimental error and is not considered to be significant.

Since only a decrease in peak area for the partially folded species is observed and no significant amount of unfolded material is present in the earliest spectrum obtained in refolding, we can conclude that conversion of the unfolded state(s) (U) into the observed partially folded state (I) is rapid compared with conversion of the intermediate state into the native state (N), i.e., eq 1, where I is the intermediate responsible for P_1–P_5.

$$U \overset{\text{fast}}{\leftrightarrow} I \overset{\text{slow}}{\leftrightarrow} N \qquad (1)$$

As can be seen from Figures 4 and 5, there is a low-intensity resonance, P_4, that is found between P_3 and the resonance from native His-48. The peak area of P_4 apparently decreases much more rapidly than those of P_1–P_3 ($t_{1/2}$ = 120 s). This resonance is probably associated with an intermediate species preceding that responsible for P_1–P_3 and P_5.

Nature of Transient Species. The most likely possiblities for the source of the new resonances observed during refolding are (1) different states of unfolded protein, (2) different forms of nativelike protein, and (3) one or more partially folded

intermediates. We can eliminate the possiblity that resonances P_1–P_5 correspond to different forms of unfolded protein on the following grounds: none of the resonances have a chemical shift corresponding to that of solvent-exposed His C2; previous investigations (Westmoreland & Matthews, 1973) have shown that the chemical shift of U is practically temperature independent; the chemical shifts of P_1–P_5 cover a wider range of values than those of native His-12, -105, and -119 (Figure 2A), implying significantly different environments for the His C2 protons responsible for the new resonances, inconsistent with unfolded protein; the selective deuteration experiments show a differential loss in area for each of the resonances, and therefore, an individual His residue must be responsible for each of the new resonances; each of the individual new resonances converts to native at the same rate; and the positions of some of the transient resonances are similar to those of partially folded intermediates observed during *unfolding* (R. G. Biringer and A. L. Fink, unpublished results).

Similarly, the above arguments, and the fact that on completion of folding a spectrum identical with that of authentic native protein is obtained, imply that the transient resonances do not arise from native protein. The possibility that they are artifacts, e.g., arising from nonequilibrated sample temperature, can be discounted. Small changes in the chemical shift of the HOD resonance are observed due to temperature equilibration during the initiation of refolding, but these are complete within 3 min.

On the other hand the evidence to support the contention that the transient resonances reflect an intermediate(s) on the folding pathway is strong. The significantly different chemical shift for these signals implies different environments for the His residues and environments that are in between those of native and unfolded states. The similarity in rate of disappearance of the transient signals to that of growth in area of the peaks from the native protein indicates the conversion (direct or indirect) of the former into the latter. Refolding monitored by the change in absorbance at 286 nm also reveals a reaction of similar rate as that seen by NMR, implying a common process that involves both Tyr and His residues in folding. That the chemical shifts of the transient species observed in unfolding are in the same region as those of partially folded intermediates detected in unfolding is also strongly suggestive of their nature. We therefore conclude that the new resonances observed during refolding correspond to one partially folded intermediate state (or set of substates, since the resolution of the data is insufficient to distinguish small changes in chemical shift during the refolding).

Assignment of Transient Resonances. When the *unfolding* of RNase A is monitored in the presence of methanol by collection of spectra of the His C2 region as the temperature is raised through the transition region, partially folded species appear in slow exchange with native protein (Figure 2D) (R. G. Biringer and A. L. Fink, unpublished results). The chemical shifts of these resonances, which have been identified by selective deuteration, show temperatures dependence, due to fast-exchange processes; consequently extrapolation of these chemical shifts to −16 °C is complicated. However, examination of the chemical shifts from the partially folded species at the lowest temperature at which they appear indicates the following limits: His-12 ≤8.55, His-105 ≥8.65, and His-48 ≥ His-119 ≥8.69 ppm. The chemical shifts for the intermediate observed in refolding are 8.42 (P_3), 8.65 (P_2), 8.70 (P_1), and 8.80 (P_5) ppm. If the partially folded intermediate(s) observed in unfolding corresponds to the one observed in the present NMR experiments, then we would assign P_5 to His-48, P_1 to His-119, P_2 to His-105, and P_3 to His-12.

Scheme I

$$U_F \longrightarrow N$$

$$U_S{}^1 \xrightarrow{k_1} N$$

$$U_S{}^2 \xrightarrow{k'_1} I \xrightarrow{k_2} N' \xrightarrow{k_3} N$$

In order to obtain an unambiguous assignment of the resonances from the partially folded material, we used a selectively deuterated sample of RNase A. This deuteration results in the reduction of the His C2 protons to the following levels when compared to His-105 C4: His-105 0.05 proton, His-12 0.12 proton, His-119 0.65 proton, and His-48 0.91 proton. Refolding of this sample in 35% methanol, pH* 2.8, at −15 °C revealed two major resonances from partially folded species at 8.80 (P_5) and 8.70 (P_1) ppm (Figure 6). On the basis of the relative peak areas, the resonances were assigned as follows: P_5 to His-48, P_1 to His-119, and P_3 to His-12. Thus P_2 presumably corresponds to His-105. In the native structure His-105 is rather exposed and has a chemical shift closest to that of fully solvent exposed His C2. It is therefore quite resonable to assign P_2, which is closest to the δ of solvent-exposed His C2, to His-105. The foregoing assignments are in agreement with those obtained from extrapolation of the chemical shift data in unfolding experiments (see above).

Characterization of Partially Folded Intermediate(s). Since the rate constant for phase 2 observed in refolding monitored by ΔA_{286} is similar to that for the major phase in the NMR experiments, it is reasonable to assign both transients to the same process, especially as there is accord between the two types of measurement on the amount of native or nativelike material present at the beginning of that stage of the refolding (~50%).

How can these experimental observations be related to the underlying molecular events? The two most likely explanations for our results are either multiple unfolded states or multiple partially folded intermediate states or a combination of these. It is quite clear that some of the unfolded material refolds rapidly (≤3 min) to native and some of the unfolded protein rapidly forms one or more partiallyfolded intermediate states.

The model that best fits the observations is shown in Scheme I. Crystallographic studies have shown that Pro-93 and Pro-114 are cis in the native state. Thus the major slow-unfolding species are expected to have either or both of these prolines in their nonnative trans configuration and Pro-42 and Pro-117 in their native trans configuration.

On the time scale of the reported NMR experiments U_F is expected to refold very fast. A minimum of two different slow-refolding species is required. In Scheme I we propose that $U_S{}^1$ refolds to the native state at a rate that corresponds to phase 1 in the absorbance experiments. The present data do not permit us to determine whether a partially folded intermediate equivalent to I is present in this reaction. The major transformation of the partially folded intermediate observed by NMR arises from a second slow-refolding species, $U_S{}^2$, and results in formation of a nativelike species (N′), with one proline in the incorrect configuration. Isomerization of this Pro to the correct configuration is responsible for the slowest phase observed in the absorbance experiments, that is, Scheme I, where k_i corresponds to the rate constants for the reactions measured by change in absorbance. The rate constants calculated for the NMR experiments (Table I, Figure 7) correspond to k_2.

Since both 2′-CMP binding data and the NMR experiments indicate native protein to be present at the end of phase 2 and

yet the absorbance changes indicate a further major reaction (phase 3) (amplitude = 30% of the total observed absorbance change), we conclude that the material formed in phase 2 is not the final native form but rather a nativelike conformation in which Pro-93 or Pro-114 is in its nonnative trans configuration, N′ (Scheme I). Thus phase 3 corresponds to the isomerization of proline to its correct configuration. Due to the proximity of Tyr-92 to Pro-93 (or Tyr-115 to Pro-114) the slightly nonnative conformation present when the proline is in the incorrect configuration is observed as a significant absorbance change due to differences in the Tyr environment, which is detected in the absorbance measurements but not in the His C2 NMR experiments.

The resonances from the partially folded material in *refolding* cover a wider range of chemical shifts than those observed in *refolding* (compare Figure 5A with Figure 2D). This suggests that the intermediate(s) observed during refolding has (have) a greater variation in His environment, presumably more nativelike, than those observed during unfolding. Thus the partially folded intermediate(s) in refolding will be more compact than those in unfolding. A compact, but nonnative form of RNase A, has been reported by Creighton (1980) from studies using area gradient gels.

Mechanistic Implications. The major points of interest from the present investigation are as follows: (1) considerable native protein is formed very rapidly even at the low temperatures used in these experiments; (2) there is a relatively compact intermediate (or intermediates) (I) that can be observed by NMR during refolding in aqueous methanol in the −10 to −20 °C region, and thus, the folding pathway must be intermediate controlled; (3) the multiphasic changes observed during refolding are due both to refolding of different configuration isomers at different rates and to multiple populated intermediate states during refolding.

It is clear from both the unfolding and refolding experiments that methanol, and to a lesser degree low temperature, stabilizes partially folded intermediate states of RNase A. The presence of a relatively hydrophobic organic cosolvent, such as methanol, is expected to stabilize structural entities that have exposed apolar surfaces. Thus we would expect methanol to stabilize the unfolded state but destabilize the native state, as observed (Figure 1). The observed stabilizing effect of methanol on the partially folded intermediate is consistent with a model in which the initially formed units of structure involve hydrogen bonding (secondary structure), which subsequently pack together, predominantly via hydrophobic interactions. This interpretation relates to events during the folding process and does not question the possible existence of hydrophobic clusters in the "unfolded" state prior to the initiation of productive folding. Confirmation that secondary structure is formed rapidly, and early, in the refolding of RNase A comes from recent circular dichroism studies (B. Lustig and A. L. Fink, unpublished resluts). These show that at pH* 3.0, −40 °C, the ellipticity at 222 nm is that of the native protein within 25 s of initiating the refolding (the effective dead time of the system).

The simplified picture that thus emerges is that the early events in the folding of RNase A in aqueous methanol involve predominantly the formation of units of secondary structure with exposed hydrophobic regions. These initially formed structural units then pack together by predominantly hydrophobic interactions.

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References

Baldwin, R. L. (1975) *Annu. Rev. Biochem. 44*, 453–475.
Baldwin, R. L. (1980) in *Protein Folding* (Jaenicke, R., Ed.) p 369–386, Elsevier/North-Holland, New York.
Baldwin, R. L., & Creighton, T. E. (1980) in *Protein Folding* (Jaenicke, R., Ed.) p 217–260, Elsevier/North-Holland, New York.
Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, p 232–242, McGraw-Hill, New York.
Blum, A. D., Smallcombe, S. H., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 305–316.
Brandts, J. F., Halvorsen, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.
Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157–6161.
Creighton, T. E. (1980) *J. Mol. Biol. 137*, 61–81.
Douzou, P. (1977) *Cryobiochemistry*, Academic Press, New York.
Fink, A. L. (1977) *Acc. Chem. Res. 10*, 233–239.
Fink, A. L., & Grey, B. L. (1978) in *Biomolecular Structure and Function* (Agris, P. F., Sykes, B., & Loeppky, R., Eds.) p 471–477, Academic Press, New York.
Fink, A. L., & Cartwright, S. J. (1981) *CRC Crit. Rev. Biochem. 11*, 145–207.
Hagerman, P. J., & Baldwin, R. L. (1976) *Biochemistry 15*, 1462–1473.
Hagerman, P. J., Schmid, F. X., & Baldwin, R. L. (1979) *Biochemistry 18* 293–297.
Henkens, R. W., Gerber, A. G., Cooper, M. R., & Herzog, W. R. (1980) *J. Biol. Chem. 255*, 7075–7078.
Howarth, O. W., & Lian, L. Y. (1981) *J. Chem. Soc., Chem. Commun.*, 258–259.
Hui Bon Hoa, G., & Douzou, P. (1973) *J. Biol. Chem. 248*, 4649–4656.
Markley, J. L. (1975) *Biochemistry 14*, 3554–3561.
Morris, R. G., Saliman, G., & Dunn, M. F. (1980) *Biochemistry 19*, 725–732.
Nall, B. T., Garel, J., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317–330.
Ptitsyn, O. B., & Rashin, A. A. (1975) *Biophys. Chem. 3*, 1–20.
Schmid, F. X. (1981) *Eur. J. Biochem. 114*, 105–109.
Schmid, F. X., & Baldwin, R. L. (1979) *J. Mol. Biol. 135*, 199–215.
Schmid, F. X., & Blaschek, H. A. (1981) *Eur. J. Biochem. 114*, 111–117.
Westmoreland, D. G., & Matthews, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 914–918.