Kopple, K. D., & Marr, D. H. (1967) *J. Am. Chem. Soc. 89*, 6193–6200.
Linderberg, J., & Michl, J. (1970) *J. Am. Chem. Soc. 92*, 2619–2625.
Liwo, A., & Ciarkowski, J. (1985) *Tetrahedron Lett. 26*, 1873–1876.
Madison, V., Young, P. E., & Blout, E. R. (1976) *J. Am. Chem. Soc. 98*, 5358–5364.
Meister, A., & Anderson, M. E. (1983) *Annu. Rev. Biochem. 52*, 711–760.
Nagaraj, R., Venkatachalapathi, Y. V., & Balaram, P. (1980) *Int. J. Pept. Protein Res. 16*, 291–298.
Prasad, B. V. V., & Balaram, P. (1984) *CRC Crit. Rev. Biochem. 16*, 307–348.
Precigoux, G., Geoffre, S., & Ourrad, E. (1986) *Acta Crystallogr., Sect. C: Cryst. Struct. Commun. C42*, 721–724.
Rao, B. N. N., Kumar, A., Balaram, H., Ravi, A., & Balaram, P. (1983) *J. Am. Chem. Soc. 105*, 7423–7428.
Ravi, A., & Balaram, P. (1983) *Biochim. Biophys. Acta 745*, 301–309.
Ravi, A., & Balaram, P. (1984) *Tetrahedron 40*, 2577–2583.
Ravi, A., Prasad, B. V. V., & Balaram, P. (1983) *J. Am. Chem. Soc. 105*, 105–109.
Robson, B., & Pain, R. H. (1971) *J. Mol. Biol. 58*, 237–259.
Rose, G. D., Gierasch, L. M., & Smith, J. A. (1985) *Adv. Protein Chem. 37*, 1–109.
Smith, J. A., & Pease, L. G. (1980) *CRC Crit. Rev. Biochem. 8*, 315–399.
Thomas, K. A., Smith, G. M., Thomas, T. B., & Feldman, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4843–4847.
Venkatachalapathi, Y. V., Prasad, B. V. V., & Balaram, P. (1982) *Biochemistry 21*, 5502–5509.
Vicar, J., Budesinsky, M., & Blaha, K. (1973) *Collect. Czech. Chem. Commun. 38*, 1940–1956.
Woody, R. W. (1973) *Tetrahedron 29*, 1273–1283.
Wüthrich, K. (1976) in *NMR in Biological Research Peptides and Proteins*, Elsevier/North-Holland, Amsterdam.
Wüthrich, K., Billeter, M., & Braun, W. (1984) *J. Mol. Biol. 180*, 715–740.
Ziegler, D. M. (1985) *Annu. Rev. Biochem. 54*, 305–329.
Zimmerman, S. S., & Scheraga, H. A. (1977) *Biopolymers 16*, 811–843.

Structural Studies of a Folding Intermediate of Bovine Pancreatic Ribonuclease A by Continuous Recycled Flow†

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Received August 18, 1987; Revised Manuscript Received November 5, 1987

ABSTRACT: A new technique, continuous recycled flow (CRF) spectroscopy, has been developed for observing intermediates of any thermally induced, reversible reaction with a half-life of 10 s or longer. The structure can be probed by any spectroscopic method which does not perturb the system. Prolonged signal acquisitions of 8 h for ribonuclease A are possible. CRF was used to investigate the structure of the slow-folding intermediates of chemically intact ribonuclease A (RNase A) during thermal unfolding/folding under acidic conditions. The following conclusions were reached on the basis of the proton nuclear magnetic resonance and far-ultraviolet circular dichroism spectra of a folding intermediate(s): (A) The conformation of the detected folding intermediate(s) is similar to that of the heat-denatured protein. There is only limited formation of new structures. (B) The N-terminal α-helix is partially stable under these conditions and is in rapid (<10 ms) equilibrium with the denatured conformation. (C) There are long-range interactions between the hydrophobic residues of the N-terminal α-helix and the rest of the protein. These interactions persist well above the melting point. (D) An aliphatic methyl group reports on the formation of a new structure(s) that lie(s) outside of the N-terminal region. (E) The structures detected in chemically modified, nonfolding forms of the RNase A are also present in the folding intermediate(s). There are, however, additional interactions that are unique to chemically intact RNase A.

Bovine pancreatic ribonuclease A (RNase A)[1] is a small digestive protein of 124 amino acids that is denatured reversibly by a variety of agents such as chemical denaturants (Garel & Baldwin, 1973; Garel et al., 1976; Schmid & Blaschek, 1981; Lin & Brandts, 1983a; Mui et al., 1985; Schmid et al., 1986) and heat (Harrington & Schellman, 1956; Hermans & Scheraga, 1961). The protein refolds spontaneously when it is returned to conditions that favor the native structure. The process must be more directed than a random sampling of all possible structures since there are too many structures to be sampled in a reasonable amount of time (Wetlaufer, 1973). Presumably, the folding is guided by the formation of local ordered structures involving short- and medium-range interactions.

† This work was supported by research grants from the National Institute of General Medical Sciences (GM-24893) and from the National Science Foundation (DMB84-01811).
‡ NIH postdoctoral fellow, 1987.

[1] Abbreviations: C-peptide, the 13 N-terminal residues of ribonuclease A; CRF, continuous recycled flow; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; HPLC, high-performance liquid chromatography; N-terminal α-helix, the first α-helix in ribonuclease A which extends from residues 3 to 13; NMR, nuclear magnetic resonance; ppm, parts per million; RNase A, bovine pancreatic ribonuclease A; S-peptide, the 20 N-terminal residues of ribonuclease A; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; $8SO_3^{2-}$-RNase A, disulfide-reduced and sulfonated bovine pancreatic ribonuclease A; T_m, midpoint of the ribonuclease unfolding transition; U_f and U_s, fast- and slow-refolding forms of unfolded ribonuclease A, respectively; UV, ultraviolet; UVCD, ultraviolet circular dichroism; 1D, one dimensional.

0006-2960/88/0427-2471$01.50/0

Unfortunately, RNase A folds in a highly cooperative manner, and it has been extremely difficult to isolate stable folding intermediates, even though the existence of specific structured intermediates has been inferred from proteolytic digestion studies (Burgess & Scheraga, 1975) and verified by Raman (Chen & Lord, 1976) and X-ray studies (Gilbert et al., 1982). Thus, little progress has been made in identifying these locally ordered structures under native conditions.

This paper described a new technique for studying kinetically unstable intermediates by spectroscopic methods. This technique is used to investigate the reversible folding pathway of RNase A from a thermally denatured state. RNase A has been selected for study because there is a large amount of literature published on its refolding pathway. Furthermore, up to 80% of the protein refolds with a first-order relaxation time of 30–300 s (Lin & Brandts, 1983a). This property makes it feasible to study the refolding of disulfide-intact RNase A using conventional mixing techniques.

Some of the details concerning the slow-folding phases of RNase A have been elucidated. In the denatured state, certain covalent bonds undergo reversible isomerization. For example, proline-93, which is all cis in the native protein, forms a mixture of cis and trans isomers when the protein is denatured (Lin & Brandts, 1983b; Schmid et al., 1986). When RNase A is jumped back into native conditions, only the protein molecules with the native (cis) isomer of proline-93 refold rapidly into the native conformation. The refolding is greatly retarded in the molecules that have the trans isomer of proline-93.

Under the conditions used in the present study, thermally induced refolding at acidic pH, the protein refolds in two separate phases. One phase is extremely fast and accounts for 20% of the protein as judged by UV absorbance. Presumably, this phase is identical with the fast phase, U_f, seen in refolding from chemical denaturants (Garel & Baldwin, 1973; Garel et al., 1976; Schmid & Blaschek, 1981; Lin & Brandts, 1983a; Mui et al., 1985; Schmid et al., 1986). This pathway has a first-order relaxation time of approximately 20 ms and cannot be monitored by our current techniques. The second phase, involving 80% of the protein, refolds with a first-order relaxation time of 30–300 s, depending on the conditions. The amplitude and relaxation time of this phase are similar to the kinetics of the slow-folding phase, U_s, seen in the refolding of RNase A from chemical denaturants (Lin & Brandts, 1983a). In experiments performed near neutral pH, this slow phase is further subdivided into U_s^I and U_s^{II} (Schmid & Blaschek, 1981; Lin & Brandts, 1983a; Mui et al., 1985; Schmid et al., 1986). Fluorescence and absorbance spectroscopy under a broad range of conditions have demonstrated that the observed changes in the optical properties of the slow phase upon refolding are dominated by two first-order processes. The results indicated that the refolding in the slow phases is inhibited by the presence of two nonnative isomers of covalent bonds. However, there is no consensus on the identity of these bonds (Lin & Brandts, 1983b, 1984; Schmid et al., 1986).

Experimental evidence indicates that structures are formed during the slow-folding phase. Blum et al. (1978) investigated the structure of a kinetically unstable folding intermediate formed by cooling thermally denatured RNase A and probing the structure by 1D proton NMR. The data were acquired by first warming a sample of RNase A above its melting point in an NMR cell and then cooling it in an ice bath, followed by placing it rapidly in the NMR probe and recording the spectrum.

The results demonstrated that one of the four histidines of RNase A was in a unique environment in the folding intermediate. Therefore, structure(s) must have formed in the intermediate which perturbed the chemical shift of this histidine. The results were consistent with the partial formation of the N-terminal α-helix (see Results for details). However, the interpretation of the data was complicated by problems with resolution, a low signal-to-noise ratio, and the absence of data at a variety of temperatures.

These difficulties could be surmounted if it were possible to trap the folding intermediate. Several groups have investigated the folding of RNase A where complete refolding was inhibited by chemical modification (Bierzynski et al., 1982; Rico et al., 1983, 1986; Kim & Baldwin,1984; Montelione et al., 1984; Swadesh et al., 1984; Haas et al., 1987). These compounds have been very useful in studying the formation of structures in specific regions of the protein, such as the N-terminal α-helix. However, these chemical modifications alter the covalent structure, which in turn perturbs the medium- and long-range interactions.

The technique presented in this report effectively traps a slow-folding intermediate(s) of RNase A. This intermediate can be studied by any spectroscopic technique even if prolonged data acquisition times are required. There are minor reductions in both sensitivity and resolution. In spite of these problems, the technique is sufficiently powerful to provide new information about the refolding of RNase A.

Concerning nomenclature, the term "refolding ensemble" refers to the large number of kinetically unstable unfolded and/or partially folded forms of RNase A that are present at short times after the initiation of refolding. The experimental evidence indicates that the ensemble includes a folding intermediate(s) and that the spectral properties of this (these) intermediate(s) are distinct from those of the heat-denatured protein. This is the folding intermediate referred to in the title of the paper. The relative proportions of the unfolded and/or partially folded forms of RNase A are influenced by temperature, and the conformations of these species interconvert rapidly on the NMR time scale (<10 ms). Therefore, the spectra represent the time-averaged properties of the different nonnative forms of the protein. Also, it should be noted that the experimental results obtained elsewhere (Lin & Brandts, 1983a,b; Mui et al., 1985; Schmid et al., 1986) indicate that the refolding ensemble should contain several different isomeric forms of RNase A which do not interconvert rapidly. However, the various isomers do not have any distinguishable spectral properties under these conditions, and the existence of such isomers will not be discussed further.

Finally, the terms U_f and U_s have been adopted from the literature describing the refolding of chemically denatured RNase A (Garel & Baldwin, 1973; Garel et al., 1976; Schmid et al., 1986). Our preliminary data from near-UV absorption experiments indicate that the relative proportions of the species are the same (within 10%) in both thermally and chemically denatured forms of the protein.

Experimental Procedures

Materials. Ribonuclease A (grade II-A, Sigma) was purified further by ion-exchange chromatography (Taborsky, 1959). Ribonuclease S-peptide (grade XII-Pe, Sigma) was used without further purification. Glycine-d_2 was a generous gift from Dr. Y. C. Meinwald. All other chemicals were analytical grade and were used without further purification.

Continuous Recycled Flow (CRF) Spectroscopy. In the continuous recycled flow experiments, protein refolding was induced by a temperature jump from 60 °C (the T_m being 42

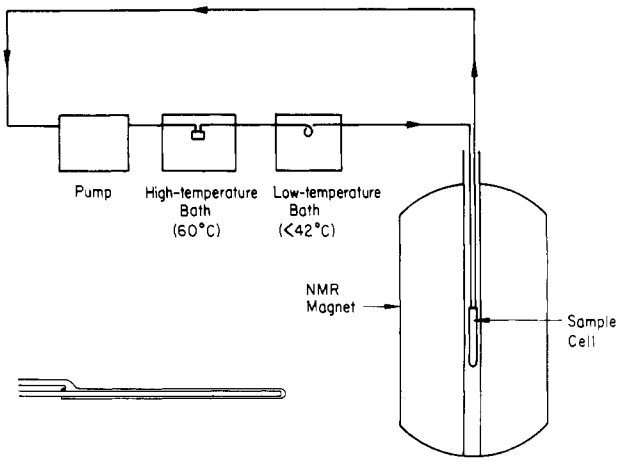

FIGURE 1: Schematic diagram for continuous recycled flow (CRF) spectroscopy. A detail of the sample cell is shown in the lower left. Initially, the protein is thermally unfolded at 60 °C. The high-temperature reservoir contains 2 mL of solution, approximately the same volume as the rest of the system. The denatured protein is pumped into 0.5-mm i.d. Teflon tubing. A loop of the tubing is submerged in the low-temperature bath. The temperature of the bath matches the temperature of the sample cell. Rapid cooling initiates protein refolding. The cooled protein is then pumped into the sample cell. The spectrum is measured, and then the protein is displaced by a new sample which is prepared by an identical cycle of heating and cooling. The protein passes through the peristaltic pump and is returned to the high-teperature reservoir. The pump is left on during spectral acquisition, and the sample in the sample cell is constantly replenished with freshly cooled RNase A from the high-temperature reservoir. The protein in the sample cell becomes "trapped" at a given point in the refolding pathway. The extent of refolding is determined by the delay time between the initiation of protein folding and the acquisition of the spectrum.

°C at pH 2.4) to a temperature below the T_m. The major advantage of this approach was that the changes in temperature did not alter the chemical composition of the protein solution. Since the thermally induced refolding of RNase A is reversible, the spent sample was recycled directly into the high-temperature reservoir without any further processing. Repeated spectral acquisitions were carried out on the same sample which was pumped continually through the closed-loop system depicted in Figure 1.

The following is a step by step description of the CRF experiments. Initially, the protein was unfolded in a high-temperature reservoir maintained at 60 °C (Figure 1). The buffer was 10 mM glycine/0.2 M NaCl, pH 2.4. The unfolded protein was then pumped through Teflon tubing (0.5-mm i.d.) that was submerged in a low-temperature bath. The low-temperature bath was set to the same temperature as the sample compartment in the spectrometer (11–37 °C, depending on the experiment). The rapid cooling initiates protein folding. The protein was then pumped into the flow-through sample cell, and the spectrum was measured. The delay time before measurement was approximately 15 s. The pump remained on while the spectra were recorded.

The protein sample in the spectrometer was stable; i.e., it remained in the same conformational state, as long as the sample was replenished constantly from the high-temperature reservoir. Therefore, the kinetically unstable intermediate(s) was (were) "trapped" at a given point of the refolding pathway. During the 15-s delay time, U_f folded to the native structure. The two slow phases, U_s^I and U_s^{II}, have half-lives on the order of a minute, and only a small percentage of the protein of these phases converted to native during the delay. The remaining protein from the slow phases, approximately 50% of the total, formed an ensemble of different conformations which included a folding intermediate(s). This mixture of interconverting species will be referred to as the refolding ensemble. If the flow was stopped, all the protein in the sample cell completely refolded to native.

The spent sample was removed from the sample cell and replaced by identically prepared protein. A key point is that the refolding was reversible under these conditions, and the chemical composition of the "used" sample was identical with the solution in the high-temperature bath. Instead of disposing of the protein, it was returned to the high-temperature reservoir, thus completing the cycle. The protein was continuously pumped through the closed loop, and it underwent 5–20 transitions during a single measurement.

A Rabbit peristaltic pump (Rainin, Woburn, MA) with 2.28-mm i.d. viton pump tubing was used to pump the protein solution. The pump rate was 2.5 mL/min.

To ensure that the unfolded protein reached equilibrium between the various isomers, the volume of the high-temperature reservoir was adjusted to match the total volume of the rest of the flow system. The rate of isomerization between U_s and U_f is accelerated at higher temperature (Lin & Brandts, 1983a; unpublished data). Therefore, the conversion of the slow phases to U_f under folding conditions was readily reversed at 60 °C. The high-temperature reservoir was also constructed to act as a bubble trap to remove air bubbles from the line.

Periodically, the protein was checked for covalent modification after use by cation-exchange HPLC analysis on an analytical Mono-S column (Pharmacia), using a range of pHs from 3 to 8. The NaCl gradients were adjusted to resolve single charge differences in the protein. Degradation products were $\leq 1\%$.

The high-temperature reservoir was equilibrated at room temperature for the control (folded) samples. The pump remained on during these measurements.

Optical Measurements. Ultraviolet circular dichroism (UVCD) measurements were made on a modified Cary-14 spectrometer. A 111-200-UV Pockel cell (Inrad; Nothvale, NJ) was installed, and the output of the machine was connected to a four-digit analogue-to-digital converter interfaced to a Prime 550 computer. All alterations on the equipment were made in this laboratory by V. G. Davenport. A type 59 MS flow cell (NSG Precision, Farmingdale, NY) with a 10-mm light path length was used. This cell had no UVCD absorbance compared to air down to 210 nm.

Near-UV absorbance and UVCD measurements were made at a protein concentration of 1 mg/mL. Protein concentrations of 40 μg/mL were used to obtain the far-UVCD spectra. Because of its large UV absorbance at lower wavelengths, glycine was omitted from the buffer in the far-UVCD measurements.

NMR Spectroscopy. All measurements were performed on a Varian XL-400 instrument. Protein concentration was 1 mM. A custom sample cell (Figure 1) was made by centering a 3-mm narrow-walled tube inside a 5-mm NMR cell. A gap of approximately 1 mm was left between the two tubes at the bottom of the cell to allow unrestricted flow of the solution. The protein was introduced through the 3-mm inner tube and removed through the 5-mm outer tube. The 5-mm NMR cell was sealed at the top, and side arms were attached for the entry and removal of the protein. The sample cell was mounted in a custom holder, which was cut to the same dimensions as the normal 5-mm spinner, and lowered into the NMR probe. The sample cell was not rotated during acquisition of the spectra.

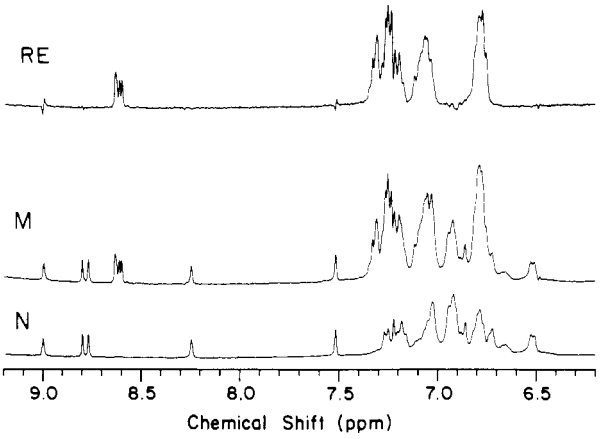

FIGURE 2: NMR spectra of RNase A at pH 2.4, 33 °C. N, native RNase A; M, the refolding mixture which contains both native RNase A and the refolding ensemble; RE, the refolding ensemble. Spectrum RE was obtained by subtracting the spectrum of native, N, properly scaled, from the spectrum of the refolding mixture, M.

D_2O (99.96% isotopic purity, Aldrich) and glycine-d_2 were used in the buffer. The pH was measured with a saturated KCl glass electrode and was not corrected for the isotope effect. The labile hydrogens of the protein were exchanged 3 times with 99.8% D_2O before use.

1D NMR measurements were made by using a single 90° pulse of 20 μs with a collection time of 2 s. A total of 512 scans were collected at a single setting. The shim settings were adjusted between each sample. A Lorentzian-to-Gaussian transformation (Ferrige & Lindon, 1978) of 5–7 Hz was used to resolve peaks and trace the temperature dependence of the chemical shifts. The spectra in Figures 2 and 3 were processed with 0.5-Hz line broadening to improve the appearance of the spectra.

Measurements were taken with and without sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal reference. When omitted, the strong singlet of $C^{\epsilon}H$ Met-13 of the unfolded protein was used as a reference. This resonance had a small linear temperature dependence which was calibrated against DSS.

Comparisons with the S-Peptide. Comparisons were made between the NMR spectra of the S-peptide, amino acids 1–20, and those of RNase A under identical conditions. The S-peptide was added as a dried powder to a solution of ribonuclease in a 1 to 1.5 molar ratio. The S-peptide had been lyophilized from an unbuffered solution at pH 2.4. A control spectrum of RNase A was measured before the addition of the peptide. The addition of the S-peptide did not affect the chemical shifts of the resolved native resonances.

Spectral Processing. During the delay before the spectra could be measured, roughly half the protein folds into the native structure. Therefore, the spectra of the refolding mixture contain resonances from both the native protein and the refolding ensemble (Figure 2). The percentage of fully folded protein was determined from the peak areas of the $C^{\epsilon}H$ His resonances in the 1D NMR spectrum. These resonances are well resolved and characterized for all forms of the protein. The concentration of each species was thereby determined, and the spectrum of the folded protein was then eliminated mathematically by subtracting a scaled spectrum of the control (native) sample.

RESULTS

Continuous Recycled Flow Spectroscopy. The proton NMR spectra of a refolding mixture of RNase A, labeled M, and the native protein, labeled N, taken at 33 °C are depicted in Figure 2. Spectrum M was acquired by using the continuous recycled flow (CRF) system. It contains additional peaks, e.g., the three resonances centered at 8.6 ppm, that are not found in the native spectrum. These peaks decrease in intensity as the protein folds to native. The spectrum of native, N, was obtained by halting the flow and allowing the protein to refold completely. The results demonstrate that the native structure is the thermodynamically stable form of the protein under these conditions. The diference between the spectra of M and N results from the presence of a kinetically unstable refolding ensemble in the sample cell. The spectrum of this ensemble, labeled RE, is shown in Figure 2. It was obtained by subtracting the native spectrum, as scaled, from that of the refolding mixture.

Spectrum M is the average of 512 transients acquired over a 20-min period. There was no measurable change in the spectrum during the course of the experiment.

Similarly, the far-ultraviolet circular dichroism (far-UVCD) spectrum of the flowing refolding mixture was distinctly different from that of the native protein (data not shown). The refolding mixture had less CD absorption than the native protein and an altered spectral shape. Once the flow was stopped, the spectrum of the native protein reappeared.

The movement of the sample through the flow cell did not distort the data in any significant manner. Optical measurements showed that the circulation caused minor drifts in the base line but no systematic errors. In NMR experiments at 11 °C, the circulating control sample had narrower line widths than the comparable nonflowing control sample at the same temperature. Presumably, this effect arose because the flow itself helped to average out the field inhomogeneity. The circulation did not change the chemical shift of any peak measurably.

There are, however, some significant problems with the CRF technique. The spectrum of the refolding ensemble, RE, was obtained by subtracting the spectrum of the control (native) sample from that of the refolding mixture (see Experimental Procedures). The relative proportions were adjusted to eliminate the spectrum of the native species. A disadvantage of this approach is that it effectively eliminates the spectrum of all nativelike species, including any intermediate that had folded into a native structure before all the peptide bonds had isomerized to the native form. The existence of such a nativelike intermediate, called I_N, has been demonstrated during refolding from chemical denaturants at neutral pH (Schmid & Blaschek, 1981). No published results indicate that such an intermediate would exist under the acidic conditions described in this paper.

The experimental results also indicate that there are no I_N-like intermediates formed under these conditions. Preliminary near-UV absorbance studies with CRF indicated that the amount of native protein at zero time is 75% ± 10%, which compares favorably with the amount of U_f (80%) found in solvent-jump experiments (Lin & Brandts, 1983a). Furthermore, the line widths of the four $C^{\epsilon}H$ His resonances were determined in the unprocessed spectra of the refolding mixture. If any I_N-like species were present in this mixture, then the line widths of the peaks probably would be broadened by chemical shift inhomogeneity. Within the accuracy of the data, ±0.001 ppm, there was no detectable change in line width compared to the control sample. This indicates that there was no second nativelike species in the solution.

Because of the arrangement of the flow system, it was impossible to spin the sample tube in the NMR spectrometer. Line widths were approximately 1 Hz broader than compa-

rable measurements made with a spinning NMR cell. The line broadening was partially reversed by processing the data with a Lorentzian-to-Gaussian transformation of 7 Hz (Ferrige & Lindon, 1978). Interpretation of the spectra was not affected significantly by the minor loss of resolution.

The subtraction of the native spectrum from that of the refolding mixture effectively doubled the noise level of the final spectrum. Therefore, longer accumulation times were required than for comparable measurements on a pure sample.

A more significant problem results from inherent limitations in subtracting one NMR spectrum from another. It was impossible to tune the field inhomogeneity reproducibly. This task was further complicated by having a nonspinning sample. As a result, there were inevitably minor changes in line shape between the two spectra which generated errors in subtraction. These errors were most pronounced for the sharpest lines.

Another source of error was the extreme sensitivity of some of the native resonances to small changes in solution conditions. This problem arose when the refolding sample did not reach thermal equilibrium in the low-temperature bath (Figure 1). Drifts of 0.5 °C or less caused measurable changes in the chemical shifts. As a result, false peaks appeared in the calculated spectra of the refolding ensemble (spectrum RE, Figure 2). These dispersive-shaped peaks, at 7.5 and 9.0 ppm, arose from subtraction errors when the chemical shifts of the native resonances were different in the control sample and in the refolding mixture. Fortunately, none of these false peaks caused significant distortions in the resonances of interest.

Protein Aggregation. A more intractable problem was caused by aggregation of the protein after prolonged recycling. This problem was observed only at the high protein concentrations (1 mM) used in the NMR experiments. As soon as the aggregation reached detectable levels, the protein solution turned into a milky white gel that was too viscous to pump through the tubing. Generally, the aggregation commenced after 8 h of recycyling, which corresponds to 200 unfolding/folding transitions, but this time was highly variable. The aggregation was thermally induced but could not be reversed by changes in temperature. The aggregate could be redissolved only in high concentrations of chemical denaturants, such as 6 M guanidine hydrochloride, at pH 8 or greater.

Experiments were performed to determine if aggregation resulted from the chemical degradation of RNase A. The redissolved aggregate was desalted by ultrafiltration to yield a soluble guanidine-free solution which was analyzed by HPLC on a Mono-S column using a range of pHs from 3 to 8. Degradation products were $\leq 1\%$. The recovered protein was fully active enzymatically. SDS–PAGE was run on the aggregated protein under reducing and nonreducing conditions. There was no detectable covalent cross-linking nor any change in molecular weight.

The NMR spectra of partially aggregated (5–50%) RNase A samples were measured under a variety of conditions. Separate spectra were taken under conditions which favored, alternately, the native structure, the refolding ensemble, and the thermally denatured protein. Comparisons were made to the initial spectrum of the same sample. The intensity of the spectrum of the aggregate was reduced roughly in proportion to the extent of aggregation. The spectra were superimposable with the control (nonaggregated) sample with the possible exception of two to six peaks. The height of these peaks had been altered by variations in the base line. Apparently, there was almost no contribution to the proton NMR spectra from the aggregate. An explanation for this is that the large molecular weight of the aggregate shortens the T_1 time to the point where the resonances are no longer observable. Although the data do not prove that the remaining soluble RNase A was chemically intact, they do prove there had been no gross conformational changes.

Preliminary experiments failed to provide an explanation for the observed aggregation. First, prolonged heating of RNase A above the melting point does not cause aggregation. The aggregation is apparently induced by the refolding process itself. Second, chemical contaminants leaching from the pump hose do not seem to be important. The substitution of a poly(vinyl chloride) pump hose had little effect.

These experiments demonstrate that the aggregation was not caused by a covalent modification of the protein. Furthermore, the NMR data indicate that the protein was not slowly converted to a second conformation which eventually precipitated. The predominant protein species in the experimental sample was native RNase A. Furthermore, recycling had no observable effect on any of the spectral properties except the intensity of the NMR peaks. In fact, all but one of the runs were made on unaggregated material, and the single run (with detectable aggregation) led to identical results. A simple interpretation is that the protein remained intact until a trace contaminant caused the rapid aggregation of the sample.

Spectroscopic Measurements of Conformation. The far-UVCD spectrum between 210 and 250 nm of the refolding mixture of RNase A was measured at 11, 21, and 33 °C. Within the accuracy of the data, the refolding spectra could be simulated by a composite spectrum of $\sim 50\%$ folded protein taken at the same temperature and $\sim 50\%$ denatured protein measured at 60 °C. The relative proportions of the two species agree favorably ($\pm 5\%$) with the corresponding values obtained by proton NMR. The data imply that the CD spectrum of the species present in the refolding ensemble is similar to the thermally denatured protein. It should be noted that the accuracy of the UVCD spectra was limited to ± 400 deg cm^2 dmol^{-1} and the partial formation of structures such as the N-terminal α-helix (see next section) may have been obscured. The error (400 deg cm^2 dmol^{-1}) represents 5–10% of the total change in UVCD absorbance observed in the thermal denaturation of RNase A, and the far-UVCD spectra were only accurate enough for qualitative interpretations.

The structural similarity between the refolding ensemble and the thermally denatured protein is also apparent in the 1D proton NMR spectrum (Figure 3). In the native protein, the compact structure affects the chemical shifts of the aromatic resonances. Each residue has a unique environment, and the peaks are found between 6.4 and 7.6 ppm (Lenstra et al., 1979; Tanokura, 1983; Howarth & Lian, 1984). In the denatured protein, the aromatic resonances become degenerate and collapse to the positions similar to those found in isolated peptides: Phe-ring, 7.34 ppm; C$^\delta$H Tyr and C$^\delta$H His, 7.15 ppm; C$^\epsilon$H Tyr, 6.86 ppm (Bundi & Wüthrich, 1979). The spectrum of the refolding ensemble bears a close resemblance to that of the denatured protein.

A more detailed examination of the NMR spectra reveals that some of the chemical shifts observed in the refolding ensemble are distinctly different from those found in the denatured protein, although the differences are small compared to those seen in the native protein. For example, one of the C$^\epsilon$H His resonances in the refolding ensemble is shifted from the unfolded positions; there are at least three resolved peaks centered at 8.6 ppm (Figure 3A) in the spectrum of the refolding ensemble compared to two in the spectrum of the unfolded protein. This indicates that a folding intermediate

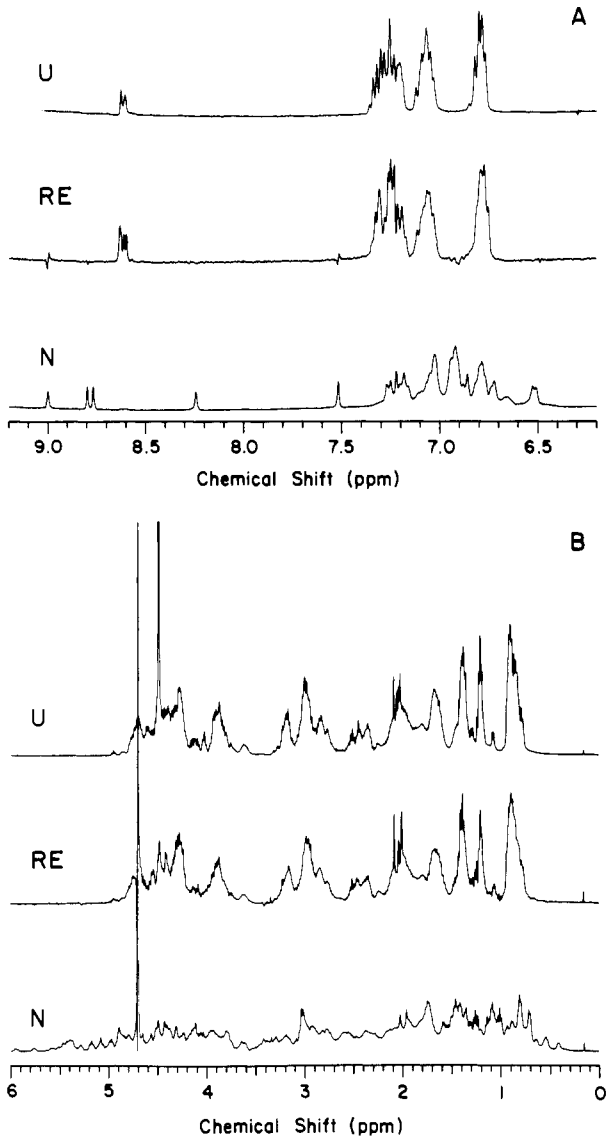

FIGURE 3: Aromatic region (A) and aliphatic region (B) of the NMR spectrum of ribonuclease A. The spectra are labeled as follows: U, unfolded protein at 53 °C; RE, the refolding ensemble at 33 °C; N, folded protein at 33 °C.

has formed under these conditions and its structure affects the chemical shifts of one of the histidines, thus breaking the degeneracies.

Temperature Dependence of Chemical Shifts. In order to examine the formation of structure in the refolding ensemble in greater detail, 1D NMR spectra of the nonnative forms of RNase A were taken from 11 to 70 °C. This approach has been used by several investigators to study the formation of compact structures in chemically modified, nonfolding forms of RNase A (Rico et al., 1983, 1986; Kim & Baldwin, 1984; Swadesh et al., 1984). The term nonnative RNase A refers to conformations found in both the refolding ensemble and the heat-denatured protein. CRF spectroscopy was used to make measurements below the midpoint of the folding transition (T_m = 42 °C). All spectra obtained at or above 42 °C were made on nonflowing, thermally denatured RNase A samples from the same preparation used in the refolding studies. These measurements were carried out with either the NMR flow cell or a standard spinning sample cell. There was no detectable change in the chemical shifts between the two sets of spectra.

A plot of the chemical shifts of the four $C^{\epsilon}H$ His resonances versus temperature appears in Figure 4A (spectrum in Figure

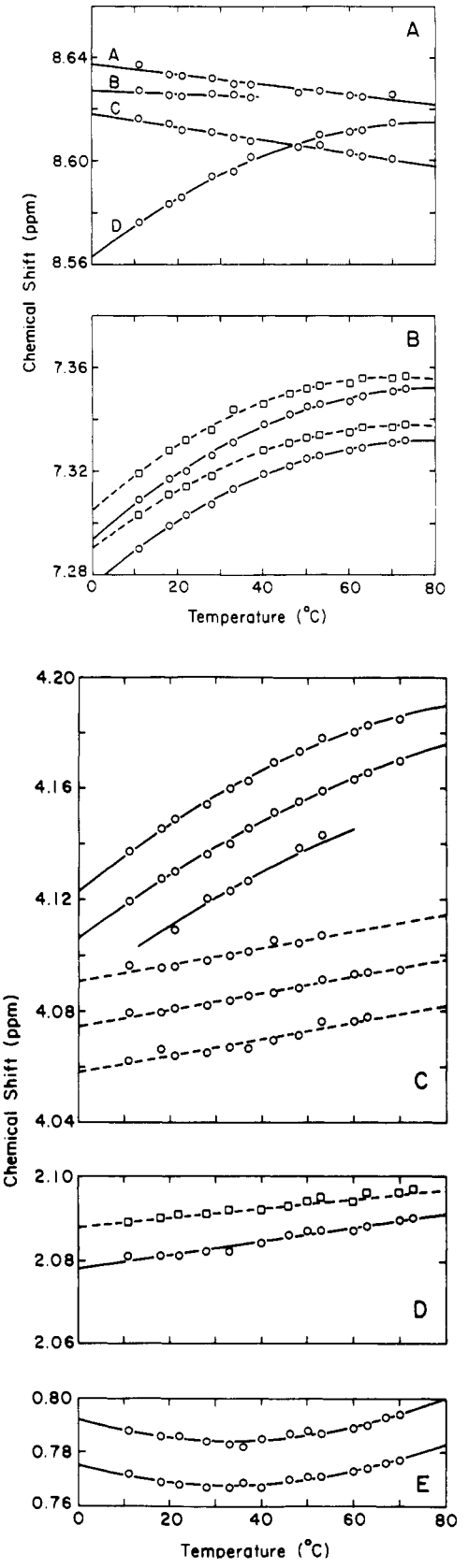

FIGURE 4: Chemical shifts of selected residues as a function of temperature. (Panel A) $C^{\epsilon}H$ His resonances: (A and B) His-48 and -119, respectively; (C) His-105; (D) His-12. (Panel B) Two peaks from the triplet of meta-ϕH Phe-8: (—) chemically intact unfolded RNase A; (---) S-peptide. (Panel C) (—) Triplet of $C^{\alpha}H$ Lys-7: (---) triplet of $C^{\alpha}H$ Lys-1. (Panel D) Singlet peak of $C^{\epsilon}H_3$ Met-13: (—) from chemically intact unfolded RNase A: (---) from S-peptide. (Panel E) Two peaks belonging to an aliphatic methyl resonance. All these peaks are displayed in Figure 5.

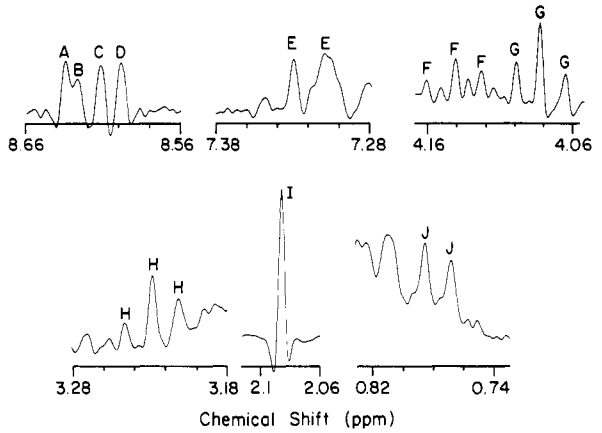

FIGURE 5: Selected resonances from the spectrum of the refolding ensemble at 33 °C. All plots, except I, are drawn to the same scale. (A and B) Singlet of $C^{\epsilon}H$ His-48 and -119; (C) singlet of $C^{\epsilon}H$ His-105; (D) singlet of $C^{\epsilon}H$ His-12; (E) two of the three peaks from the triplet of meta-ϕH Phe-8; (F) triplet of $C^{\alpha}H$ Lys-7; (G) triplet of $C^{\alpha}H$ Lys-1; (H) triplet of $C^{\delta}H_2$ Arg-10; (I) singlet of $C^{\epsilon}H_3$ Met-13; (J) doublet of unknown aliphatic methyl peak.

5). The chemical shifts of two of the peaks, labeled A and C, vary linearly with temperature. The simplest interpretation is that the alterations in the chemical shifts arise from the changes in the solvation of the imidazole group. Hence, the perturbation(s) of the chemical shifts result(s) from highly localized effects and do not reflect any changes in structure.

This evidence does not preclude the formation of local structures around these groups. It is possible that the formation of these structures does not perturb the chemical shifts. An alternative explanation is that the amount of local structure varies linearly with temperature. That would imply a noncooperative formation of structure and/or a multistate equilibrium.

The resonance labeled B cannot be detected above the T_m. The "disappearance" of this peak probably results from overlapping of resonances instead of structural changes. Simple linear extrapolation of the chemical shift indicates that this resonance overlaps resonance A above the T_m. This, combined with insufficient resolution, explains the disappearance of the peak.

Finally, the chemical shift of resonance D demonstrates a sharp rise with temperature below the T_m, followed by a pronounced leveling above the T_m. The nonlinearity of the chemical shift suggests that there is interplay between a number of factors that affect the environment of the proton. This is a strong indication of the formation of local structure.

Furthermore, the continuity of the chemical shift of resonance D, combined with the narrow line width, indicates that the local structure is in rapid exchange with a second environment. Judging from the temperature dependence of peak D above the T_m, the second environment is the unfolded protein. The position of the peak below the T_m represents the weighted average of the two conformations. If the rate of exchange between the two structures were slow on the NMR time scale (>10 ms), the peak would be broadened. At very slow exchange rates (>500 ms), there would be two distinct peaks which vary in height as the equilibrium shifts between folded and unfolded.

Experimental results from a combined sample of RNase A and the S-peptide, residues 1–20, demonstrated that resonance D belongs to the $C^{\epsilon}H$ of His-12 (see following section). The chemical shift of peak C is very similar to the chemical shift of $C^{\epsilon}H$ His-105 in disulfide-reduced and sulfonated RNase A (Swadesh et al., 1984). The $C^{\epsilon}H$ His resonances of sulfonated RNase A ($8SO_3^{2-}$-RNase A) were assigned by selective deuteriation procedures. On the basis of the same type of comparison with $8SO_3^{2-}$-RNase A, resonances A and B belong to His-48 and His-119, respectively. Peak B was not resolved in the spectra of $8SO_3^{2-}$-RNase A, and its assignment is less certain.

Our results are consistent with the partial folding of the N-terminal α-helix. This helix extends from residues 3 to 13 in the native protein. The folding of the helix has been studied in several chemically modified forms of RNase A that cannot fold. Examples include the isolated C-peptide, residues 1–13 (Bierzynski et al., 1982), the isolated S-peptide, residues 1–20 (Silverman et al., 1972; Rico et al., 1983, 1986; Kim & Baldwin, 1984), and $8SO_3^{2-}$-RNase A (Swadesh et al., 1984). These studies demonstrate that the helix is partially stable under conditions where chemically intact RNase A is folded. The partial formation of this helix has also been detected in chemically intact RNase A that was unfolded in various concentrations of guanidine hydrochloride (Bierzynski & Baldwin, 1982).

As has been pointed out elsewhere (Rico et al., 1983; Swadesh et al., 1984), the folding of the N-terminal α-helix accounts for the observed changes in the $C^{\epsilon}H$ His-12 resonance. In this α-helix, the imidazole ring of His-12 stacks against the aromatic side chain of Phe-8. As the temperature decreases, the helix becomes more stable, and the phenylalanine ring current causes an increased upfield shift of $C^{\epsilon}H$ His-12. This ring-stacking interaction also causes an upfield shift of the aromatic resonances of Phe-8 (Rico et al., 1983). Above the T_m, the N-terminal α-helix is melted out, and the chemical shift of $C^{\epsilon}H$ His-12 approaches that of the other histidines.

Comparisons between the S-Peptide and RNase A. A direct comparison was made of the transient structures formed in the S-peptide, residues 1–20, and those formed in nonnative RNase A. The S-peptide, 0.7 mM, was added to a 1 mM solution of RNase A, pH 2.4, and proton NMR spectra were taken of the combined sample from 11 to 73 °C (see Experimental Procedures). Any difference in the chemical shift of two corresponding protons in each protein would be a clear indication of long-range interactions that extended beyond the end of the S-peptide. For example, intact RNase A eventually refolds to the native structure. The long-range interactions present in the native structure alter the chemical shifts by 0.2 ppm or greater. Since the side chains of the S-peptide remain exposed to solvent, the observed chemical shifts of this peptide show only small deviations from the values observed in the statistical coil (Gallego et al., 1983).

Control spectra of RNase A were recorded before the addition of the S-peptide. Subtraction of the control spectrum from that of the combined solution reproduced the spectrum of the isolated S-peptide (Figure 6). There were, however, small residual peaks (e.g., at 6.8 ppm) left from the resonances of the intact protein. These were caused by errors in subtraction arising from minor changes in line shape. The heights of the residual peaks were approximately 5–10% of the original intensity.

Assignments of the resonances of the first 20 residues in the nonnative forms of RNase A were based on the spectra of the combined solution of intact RNase A and the S-peptide. The majority of the resonances of S-peptide overlap with the corresponding protons of the nonnative RNase A. In the few cases where the peaks did not overlap, identification was based on the similarity of coupling patterns and the close proximity of the two peaks at high temperature. No assignments were

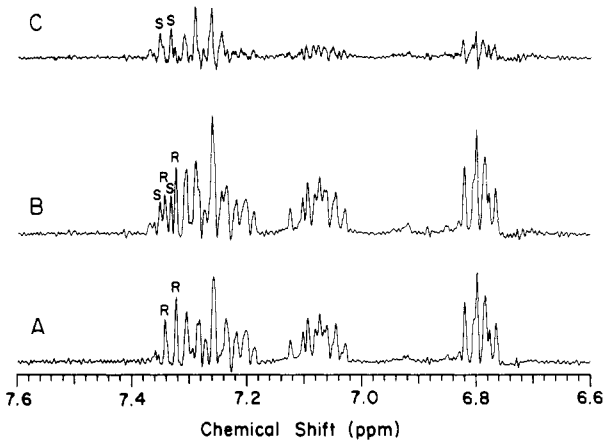

FIGURE 6: (A) Control spectrum of heat-denatured RNase A, 1 mM, at 46 °C, pH 2.4. (B) Spectrum of the same sample after addition of 0.7 mM S-peptide. (C) Result of the subtraction of (A) from (B). The intense peaks in spectrum C are from the Phe-8 resonances of the S-peptide. The two peaks in spectra B and C labeled "S" belong to the meta-ϕH Phe-8 of the S-peptide. The peaks marked with an "R" in spectra A and B belong to meta-ϕH Phe-8 of heat-denatured RNase A.

made where the individual peaks were not well resolved. Therefore, with the exception of Lys-1 and Lys-7, the $C^{\alpha}H$ resonances have not been identified.

The original resonance assignments for the S-peptide (Gallego et al., 1983) were based on "characteristic shifts, spectral analysis, double resonance, titration shifts and comparisons with the spectra of the C-peptide". No attempt was made to verify these assignments independently in the nonnative forms of RNase A. The absence of compact structure in the nonnative species causes massive overlaps in the resonances which were not resolved in 2D NMR spectra (unpublished results). It should be noted that the assignments of several of the important side chain resonances in the S-peptide, such as the meta-ϕH Phe-8, $C^{\epsilon}H$ His-12, $C^{\epsilon}H_3$ Met-13, and the $C^{\beta}H$ and $C^{\beta'}H$ of the three serines that lie outside the helix, are based upon the unique occurrence of these residues in the S-peptide.

The results indicated that most of the peaks of nonnative intact RNase A that have a nonlinear dependence of chemical shift on temperature were directly attributable to residues of the N-terminal α-helix. The plots for two such resonances, the meta-ϕH of Phe-8 and $C^{\alpha}H$ Lys-7, are shown in Figure 4B,C. The data for $C^{\alpha}H$ Lys-1 have been included for comparison. Lys-1 lies outside of the helix, and the $C^{\alpha}H$ resonance does not sense the formation of any new structures.

Furthermore, preliminary evidence indicates that there is no difference in the chemical shifts of the following resonances of the S-peptide and the refolding ensemble: $C^{\alpha}H$ and $C^{\delta}H_2$ of Lys-1; $C^{\gamma}H_2$ Glu-2; $C^{\gamma}H_3$ Thr-3; $C^{\alpha}H$, $C^{\delta}H_2$, and $C^{\epsilon}H_2$ of Lys-7; $C^{\gamma}H_2$ Glu-9; $C^{\gamma}H_2$ Gln-11; $C^{\beta}H$ and $C^{\beta'}H$ of Ser-15 and Ser-16; $C^{\gamma}H_3$ Thr-17; and $C^{\beta}H_2$ and $C^{\beta'}H$ of Ser-18. One of the resonances of the five $C^{\beta}H_3$ Ala, residues 3, 4, 5, 19 and 20, is different. It probably belongs to the C-terminal alanine (residue 20); the chemical shift of the $C^{\beta}H_3$ resonance is altered by the α-COO^- group. Differences smaller than 0.008 ppm were not resolved.

The chemical shifts of four sets of protons were distinct in the isolated fragment and intact protein. These were meta-ϕH Phe-8, $C^{\delta}H_2$ Arg-10, $C^{\epsilon}H$ His-12, and $C^{\epsilon}H_3$ Met-13. These resonances in the spectrum of the refolding ensemble were all shifted upfield from those of the S-peptide.

Above the melting point, standard NMR cells were used for the experiments in order to improve the sensitivity and resolution of the data. This made it possible to identify the resonances of nearly all of the side chain protons. With the exception of three resonances, the peaks of the S-peptide overlapped the corresponding native resonances above 42 °C. The three exceptions were meta-ϕH Phe-8, $C^{\epsilon}H_3$ Met-13, and at least one $C^{\beta}H_3$ of alanine, presumably the C-terminal residue in the S-peptide. Differences of 0.005 ppm or greater were resolved.

The chemical shift of meta-ϕH Phe-8 and $C^{\epsilon}H_3$ Met-13 (Figure 4B,D) varied smoothly with temperature from 11 to 70 °C, thus indicating a rapid equilibrium (<10 ms) between states. Furthermore, above the melting point, it was possible to compare line widths for the S-peptide and intact protein for both these resonances. There were no measurable differences to within 0.05 Hz. By comparison, the $C^{\epsilon}H_3$ resonances of methionines-29, -30, and -79 were broader than $C^{\epsilon}H_3$ Met-13 by 0.1–0.8 Hz depending on temperature. This indicates that the structures surrounding these residues in the unfolded protein did not inhibit the rotational freedom of the side chains significantly.

Two- and four-fold dilution of the combined sample had no effect on the chemical shifts of any of the resonances. This indicates that the protein was not aggregated. The results of the line-width analysis, smooth variation of chemical shift with temperature, and the lack of any visible light scattering from the sample also indicate that the protein was not aggregated under these conditions.

Other Relevant Peaks. The chemical shifts of two peaks centered at 0.79 ppm had nonlinear temperture dependence (Figure 4E). These resonances are interesting for several reasons. First, the peaks were shifted upfield by at least 0.09 ppm from their expected positions based on the chemical shift of aliphatic methyls in statistical coils (0.88 ppm for $C^{\epsilon}H_3$ Ile, 0.90 ppm for $C^{\delta}H_3$ Leu, and 0.94 ppm for $C^{\gamma}H_3$ Val; Bundi & Wüthrich, 1979). Second, the upfield shift was most pronounced at 33 °C. Finally, there are no long-chain aliphatic residues in the N-terminal α-helix. The first long-chain aliphatic residue in the sequence of RNase A is Leu-35, which does not interact with the aromatic groups of the N-terminal α-helix in the native protein.

Two peaks centered at 0.79 ppm were also observed in the 300-MHz spectrum of reduced and sulfonated RNase A ($8SO_3^{2-}$-RNase A), at t = 21 °C, pH 3.0 (G. T. Montelione, M. Adler, and H. A. Scheraga, unpublished results). The splitting of the peaks in both chemically intact and $8SO_3^{2-}$-RNase A indicates that these peaks belong to a single resonance that is coupled by 6 Hz to one proton. The upfield shift was most pronounced at 50 °C for the peaks in $8SO_3^{2-}$-RNase A, compared to 30 °C for intact RNase A.

Other Experimental Results. The NMR spectrum of the refolding ensemble was also measured at 11 °C using slower pump speeds. The slower speeds increased the delay between the initiation of folding and the acquisition of the data from 15 to 75 s. As expected, the intensity of the peaks from the refolding ensemble decreased, and the native peaks increased in intensity with longer delay times. There were, however, no detectable differences in the chemical shifts of the resonances from the refolding ensemble with a 5-fold increase in the delay time. Apparently, structures found in the refolding ensemble form a metastable equilibrium mixture until the incorrect isomer reisomerizes to the native form; thereupon, the folding is too rapid to be monitored.

Finally, the near-ultraviolet absorbance and circular dichroism of the refolding ensemble were measured at several temperatures. The spectra from 11 to 33 °C resembled those

of heat-denatured RNase A at 60 °C except for minor perturbations (data not shown). Small changes in the near-ultraviolet spectra were expected on the basis of the NMR data; i.e., most of the tyrosine ring protons had a linear change in chemical shift with temperature. Therefore, there is no unambiguous interpretation of the near-UV spectra.

Discussion

Continuous Recycled Flow Spectroscopy. This technique can be applied to any thermally induced, reversible reaction. The delay time could be reduced significantly by locating the cooling loop as close as possible to the sample cell. (Because of the narrow bore of the NMR sample tube, we have not adapted this approach to our system, but this modification could be used in Raman or CD spectroscopy.) This simple modification would reduce the delay time to about a second, beyond which the relatively low thermal conductivity of water would hamper further reductions.

A variety of spectrometers were used. No alteration of the spectrometers was necessary, and the flow system was quickly adapted by simply changing the sample cell. In general, continuous recycled flow is adaptable to any method of structure determination which does not alter the chemical composition of the sample.

A major advantage of this technique is that it significantly reduces the sample requirements compared to the standard continuous flow techniques where the spent sample is discarded. If the protein were not recycled, 1 g of RNase A would have been consumed in a single NMR measurement.

The thermally nonreversible aggregation of RNase A in one of the experiments is a source of concern, even though it does not appear to have affected the results. The chemical analysis, combined with the NMR spectra of the aggregated protein, indicates that the predominanant form of protein in these samples was still intact RNase A. The aggregation may have been initiated by trace amounts of a contaminant, possibly a partially denatured form of ribonuclease, which acted as a nucleus for aggregation.

However, the aggregation would interfere with the prolonged signal acquisitions that are required for high-resolution 2D NMR spectroscopy. Presumably, the protein would be more stable closer to neutral pH. However, the results of solvent-jump experiments (Lin & Brandts, 1983a) indicate that changes in the pH would have a significant affect on the refolding pathway.

Comparisons to Chemically Modified RNases. The results obtained here for chemically intact ribonuclease are very similar to those obtained elsewhere for nonfolding forms of the protein, such as the S- and C-peptides (Bierzynski et al., 1982; Rico et al., 1983, 1986; Kim & Baldwin, 1984) and $8SO_3^{2-}$-RNase A (Swadesh et al., 1984). The formation of the same local ordered structures, i.e., the N-terminal α-helix, was detected in all the nonfolding forms of the protein under conditions where native RNase A is stable. Data presented here confirm that these transiently stable structures are formed in the native refolding pathway.

The nonfolding forms of the protein offer important experimental advantages (Montelione et al., 1984; Swadesh et al., 1984). Certainly, it is easier to work with a stable sample than a transient folding intermediate. Furthermore, NMR spectra of isolated fragments are much less complex, thereby simplifying the task of making resonance assignments.

There are, however, compelling reasons for studying the folding intermediate in the intact protein. For example, long-range interactions cannot be studied in short isolated fragments. There are also some measurable differences in the spectra of $8SO_3^{2-}$-RNase A and intact RNase A (G. T. Montelione, M. Alder, and H. A. Scheraga, unpublished results). This report demonstrates the utility of combining both approaches.

Refolding of RNase A. Below the melting point, the N-terminal α-helix is partially formed in the isolated C- and S-peptides (Bierzynski et al., 1982; Rico et al., 1983, 1986; Kim & Baldwin, 1984), in $8SO_3^{2-}$-RNase A (Swadesh et al., 1984), and in the refolding ensemble. Most of the side chain protons of this helix have identical chemical shifts in the S-peptide and in the folding intermediate. These results indicate that there are little or no differences in relative stabilities of the α-helix in the S-peptide compared to the refolding ensemble. In particular, the similarity of the chemical shifts of the side chain protons of Ser-15, Ser-16, Thr-17, and Ser-18 demonstrates that there is no detectable formation of structure among these residues on the C-terminal side of the N-terminal α-helix.

There were, however, four protons whose chemical shifts were altered from those in the S-peptide by intramolecular long-range interactions in the refolding ensemble: meta-ϕH Phe-8, $C^{\delta}H_2$ Arg-10, $C^{\epsilon}H$ His-12, and $C^{\epsilon}H_3$ Met-13. Above the melting point, these chemical shift differences persisted for the protons meta-ϕH Phe-8 and $C^{\epsilon}H_3$ Met-13.

Phe-8 and Met-13 are by far the two most hydrophobic residues in the N-terminal region. Even at 70 °C, the chemical shifts of the side-chain protons do not collapse to the values for the same residues in the S-peptide (Figure 4B,D). The line widths of these resonances are identical with those of corresponding protons in the S-peptide, which demonstrates that the motion of the residues is not restricted by any compact structure. Judging by the linearity of the chemical shift of $C^{\epsilon}H_3$ Met-13, these protons do not participate in any cooperative formation of new structures.

These results are consistent with the condensation of some sort of hydrophobic pocket in the unfolded protein. Essentially, this pocket becomes a second solvent into which the hydrophobic residues, Phe-8 and Met-13, partition. The formation of such a hydrophobic pocket, which is guided by the side chains, would be one of the earliest events in refolding. It may provide a loosely formed core structure which guides the subsequent folding of the backbone and probably compensates for the entropy losses associated with the later stages of refolding.

Interpretation of the chemical shifts of $C^{\delta}H_2$ Arg-10 and $C^{\epsilon}H$ His-12 is more difficult. The observed upfield shift of these protons in the intermediate compared to the S-peptide may result from charge repulsion effects. Both these protons are within one bond of a positive charge. The intact protein carries 15 positive charges outside of the first 20 residues under acidic conditions. The upfield shift in these resonances would be expected as the protons are drawn close to the rest of the protein. The partial formation of the N-terminal α-helix, coupled to the close proximity of these protons to the hydrophobic residues, could force the charged side chains of Arg-10 and His-12 to interact with the rest of the protein.

The behavior of the resonances centered at 0.79 ppm is indicative of some kind of cooperative formation of structure that lies outside of the N-terminal region (Figure 4E). On the basis of theoretical calculations on benzene (Johnson & Bovey, 1958), the protons may be within 5 Å of an aromatic group. The aromatic ring current dominates the temperature dependence of the chemical shift. The fact that the upfield shift is attenuated at low temperature suggests that the interaction is entropically driven. This would be distinct from

the folding of the N-terminal α-helix which is driven by enthalpy (Rico et al., 1986).

The results presented in this paper demonstrate the formation of local ordered structures in a folding intermediate, detected in the refolding ensemble under conditions where native RNase A is stable. However, the structures seem to involve a limited number of residues. It appears that the folding intermediate is much more similar in structure to the heat-denatured protein than to the native form.

The slow-folding intermediate cannot refold completely because it contains a limited number of nonnative isomers. The kinetic data on the refolding of RNase A under a variety of conditions indicate that the bulk of the protein has no more than two incorrect isomers (Schmid & Baldwin, 1978; Schmid & Blaschek, 1981; Lin & Brandts, 1983a; Mui et al., 1985; Schmid et al., 1986). These isomers do not alter the net charge and cannot have any substantial effect on the structure of the unfolded protein beyond their immediate environment. The nonnative isomers must disrupt crucial long-range interactions that stabilize local ordered structures. Furthermore, these local ordered structures have little independent stability of their own.

In general, it is dangerous to overinterpret 1D NMR spectra of a protein with more than 600 protons. Few of the resonances are assigned, and there are significant problems with overlapping peaks. Essentially, the spectrum is litle more than a fingerprint of the protein. However, the fingerprint of the folding intermediate closely resembles that of the thermally denatured protein. The temperature sensitivity of most of the peaks is a simple linear extrapolation from thermally unfolded RNase A. This, combined with the far-UVCD, indicates that there is little formation of stable compact structures in the folding intermediate. Even 20 °C below the melting point, the structures that do exist are unstable and in rapid equilibrium with other, presumably unfolded, conformations.

ACKNOWLEDGMENTS

We thank Z. V. Franck and D. M. Rothwarf for most of the chemical analyses and purification of RNase A, D. J. Fuller and D. M. Rice for help with the NMR experiments, and V. G. Davenport, M. E. Denton, P. J. Milburn, G. T. Montelione, F. Ni, D. M. Rothwarf, and T. W. Thannhauser for helpful discussions.

Registry No. RNase, 9001-99-4.

REFERENCES

Bierzynski, A., & Baldwin, R. L. (1982) *J. Mol. Biol. 162*, 173–186.

Bierzynski, A., Kim, P. S., & Baldwin, R. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2470–2474.

Blum, A. D., Smallcombe, S. H., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 305–316.

Bundi, A., & Wüthrich, D. (1979) *Biopolymers 18*, 285–297.

Burgess, A. W., & Scheraga, H. A. (1975) *J. Theor. Biol. 53*, 403–420.

Chen, M. C., & Lord, R. C. (1976) *Biochemistry 15*, 1889–1897.

Ferrige, A. G., & Lindon, J. C. (1978) *J. Magn. Reson. 31*, 337–340.

Gallego, E., Herranz, J., Nieto, J. L., Rico, M., & Santoro, J. (1983) *Int. J. Pept. Protein Res. 21*, 242–253.

Garel, J.-R., & Baldwin, R. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3347–3351.

Garel, J.-R., Nall, B. T., & Baldwin, R. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1853–1857.

Gilbert, W. A., Lord, R. C., Petsko, G. A., & Thamann, T. J. (1982) *J. Raman Spectrosc. 12*, 173–179.

Haas, E., Montelione, G. T., McWherter, C. A., & Scheraga, H. A. (1987) *Biochemistry 26*, 1672–1683.

Harrington, W. F., & Schellman, J. A. (1956) *C. R. Trav. Lab. Carlsberg, Ser. Chim. 30*, 21–43.

Hermans, J., Jr., & Scheraga, H. A. (1961) *J. Am. Chem. Soc. 83*, 3283–3292.

Howarth, O. W., & Lian, L. Y. (1984) *Biochemistry 23*, 3515–3521.

Johnson, C. E., Jr., & Bovey, F. A. (1958) *J. Chem. Phys. 29*, 1012–1014.

Kim, P. S., & Baldwin, R. L. (1984) *Nature (London) 307*, 329–334.

Lenstra, J. A., Bolscher, G. J. M., Stob, S., Beintema, J. J., & Kaptein, R. (1979) *Eur. J. Biochem. 98*, 385–397.

Lin, L.-N., & Brandts, J. F. (1983a) *Biochemistry 22*, 564–573.

Lin, L.-N., & Brandts, J. F. (1983b) *Biochemistry 22*, 559–563.

Lin, L.-N., & Brandts, J. F. (1984) *Biochemistry 23*, 5713–5723.

Montelione, G. T., Arnold, E., Meinwald, Y. C., Stimson, E. R., Denton, J. B., Huang, S.-G., Clardy, J., & Scheraga, H. A. (1984) *J. Am. Chem. Soc. 106*, 7946–7958.

Mui, P. W., Konishi, Y., & Scheraga, H. A. (1985) *Biochemistry 24*, 4481–4489.

Rico, M., Nieto, J. L., Santoro, J., Bermejo, F. J., Herranz, J., & Gallego, E. (1983) *FEBS Lett. 162*, 314–319.

Rico, M., Santoro, J., Bermejo, F. J., Herranz, J., Nieto, J. L., Gallego, E., & Jiménez, M. A. (1986) *Biopolymers 25*, 1031–1053.

Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764–4768.

Schmid, F. X., & Blascheck, H. (1981) *Eur. J. Biochem. 114*, 111–117.

Schmid, F. X., Grafl, R., Wrba, A., & Beintema, J. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 872–876.

Silverman, D. N., Kotelchuck, D., Taylor, G. T., & Scheraga, H. A. (1972) *Arch. Biochem. Biophys. 150*, 757–766.

Swadesh, J. K., Montelione, G. T., Thannhauser, T. W., & Scheraga, H. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4606–4610.

Taborsky, G. (1959) *J. Biol. Chem. 234*, 2652–2656.

Tanokura, M. (1983) *J. Biochem. (Tokyo) 94*, 51–62.

Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697–701.