Smith, H. G., Jr., & Litman, B. J. (1982) *Methods Enzymol. 81*, 57–61.
Sobue, K., Morimoto, K., Kanda, K., Fukunaga, K., Miyamoto, E., & Kakinchi, S. (1982) *Biochem. Int. 5*, 503–510.
Stephens, R. E. (1975) *Anal. Biochem. 65*, 369–379.
Timasheff, S. N., & Grisham, L. M. (1980) *Annu. Rev. Biochem. 49*, 565–591.
Watterson, D. M., Burgess, W. H., Lukas, T. J., Iverson, D., Marshak, D. R., Schleicher, M., Erickson, B. W., Fok, K.-F., & Van Eldik, L. J. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 16*, 205–226.
Woodruff, M. L., & Bownds, M. D. (1979) *J. Gen. Physiol. 73*, 629–653.
Yamazaki, A., Uchida, S., Stein, P. J., Wheeler, G. L., & Bitensky, M. W. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 16*, 381–392.
Yau, K.-W., McNaughton, P. A., & Hodgkin, A. L. (1981) *Nature (London) 292*, 502–505.
Yee, R., & Liebman, P. A. (1978) *J. Biol. Chem. 253*, 8902–8909.
Yoshikami, S., & Hagins, W. A. (1971) *Biophys. J. 11*, 47a.
Yoshikami, S., Robinson, W. E., & Hagins, W. A. (1974) *Science (Washington, D.C.) 185*, 1176–1179.
Zimmerman, W. F., & Godchaux, W., III (1982) *Methods Enzymol. 81*, 52–57.

Characterization of the Unfolding of Ribonuclease A in Aqueous Methanol Solvents†

Anthony L. Fink* and Brooks Painter
Department of Chemistry, University of California, Santa Cruz, California 95064
Received August 18, 1986; Revised Manuscript Received November 3, 1986

ABSTRACT: The effect of methanol on the thermal denaturation of ribonuclease A has been investigated over the −40 to 70 °C range. The transition was fully reversible to at least 60% (v/v) methanol at an apparent pH of cryosolvent (pH*) of 3.0 and was examined at methanol concentrations as high as 80%. The unfolding transition, as monitored by absorbance change at 286 nm, became progressively broader and occurred at increasingly lower temperatures as the alcohol concentration increased. In 50% methanol, increasing the pH* from 2 to 6 shifted the transition to higher temperature. A substantial decrease in cooperativity was noted at the more acidic conditions. On the other hand, increasing concentrations of guanidine hydrochloride in 50% methanol caused the transition to shift to lower temperatures with little effect on the cooperativity. The observed effects on the cooperativity of the unfolding transition suggest that methanol and lower temperatures may increase the concentration of partially folded intermediate states in the unfolding of ribonuclease. Comparison of the transition in 50% methanol as determined by absorbance or fluorescence, which monitor the degree of exposure of buried tyrosines and hence the tertiary structure, to that determined by far-UV circular dichroism, which monitors secondary structure, indicated that the major unfolding transition occurred at a higher temperature in the latter case. Thus, the tertiary structure is lost at a lower temperature than the secondary structure. This observation is consistent with a model of protein folding in which initially formed regions of secondary structure pack together, predominantly by hydrophobic interactions, to give the tertiary structure. Carboxymethylated, disulfide-reduced ribonuclease was used as a model to determine the effects of methanol and temperature on unfolded ribonuclease.

Many important questions regarding the mechanism of protein folding, and the stability of the native state, remain unanswered. Although it is clear that the amino acid sequence and interactions of the polypeptide with its solvent environment determine the three-dimensional conformation of the protein, the particular factors that determine the folding pathway (in other words the "code" or "rules" for folding) are as yet unknown. Recent results are consistent with the folding pathway involving intermediate, partially folded states (Kim & Baldwin, 1982). In order to shed light on the mechanisms of folding, it will be necessary to obtain information about intermediate partially folded states, especially structural characterization, kinetics of intermediate transformation, and effects of environmental factors, including solvent, pH, and temperature, on the thermodynamic stability of the intermediates.

The native states of proteins are only marginally more stable than the unfolded state; typically the native state is 5–15 kcal mol^{-1} more stable. Under physiological conditions, the lifetimes of intermediates in folding are very short and their concentrations very low. As a consequence, most single domain proteins exhibit adherence to a two-state system, N ↔ U, under equilibrium conditions, due to the low population of the intermediate states. Consequently, it is necessary to use experimental conditions far from the physiological to stabilize and populate partially folded intermediate states for the time periods required to obtain high-resolution structural information. One such approach is to use subzero temperatures, which should permit dramatic decreases in the rates of folding.

Several recent investigations suggest that secondary structure may form early in folding (Kim & Baldwin, 1982). If this is the case, then partially folded intermediates would be expected in which the nonpolar side chains would be exposed

† This research was supported in part by a grant from the National Science Foundation and by funds from the Faculty Research Committee of the University of California, Santa Cruz.

0006-2960/87/0426-1665$01.50/0

to solvent. Since low temperatures strengthen hydrogen bonds and weaken the contribution of hydrophobic interactions, such intermediates would be expected to be stabilized at low temperatures relative to both native and unfolded states. In order to be able to work at suitably low temperatures, it is necessary to utilize a cryosolvent. We have chosen aqueous methanol mixtures, which have low viscosities and low freezing points. The presence of the relatively more hydrophobic cosolvent would be expected to stabilize preferentially the partially folded intermediates, with their exposed nonpolar residues, compared to the situation in aqueous solution.

Prior to a detailed study of the folding process at low temperature it is essential to know the effects of cosolvent concentration, pH*,[1] ionic strength, and denaturant concentration on the reversible thermal denaturation, as well as a comparison of the reaction in aqueous solution with that in cryosolvent. Ribonuclease A was the protein of choice for our initial investigation for several reasons, among them the fact that it is very soluble, it has little tendency to aggregate when unfolded, it has been shown to be catalytically functional in methanol cryosolvents (A. L. Fink, D. Kar, and R. Kotin, unpublished results), and its folding has previously been extensively studied in aqueous solution from the thermodynamic, kinetic, and structural point of view.

In this investigation, we have mapped out the position of the thermal denaturation as a function of methanol concentration and, for 50% (v/v) methanol, have determined the effects of pH* and guanidine hydrochloride (Gdn·HCl) concentration on the transition. Such information is necessary for choosing conditions for studying the kinetics of folding and unfolding in cryosolvent (R. G. Biringer and A. L. Fink, submitted for publication). The results have revealed a number of interesting features regarding protein stability and folding in the presence of cryosolvent.

Materials and Methods

Materials. Bovine pancreatic ribonuclease A was purchased from Calbiochem or Sigma (chromatographically pure grade) and further purified by chromatography on Sephadex SPC-25 (Biringer & Fink, 1982a). The concentration of RNase A stock solutions was determined from $\epsilon_{278} = 9800\ M^{-1}\ cm^{-1}$. Reduction and carboxymethylation of ribonuclease was carried out by standard procedures (White, 1967; Takahashi et al., 1977). Ultrapure Gdn·HCl (Research Plus Laboratories or Schwarz/Mann) was used; all other chemicals were of analytical reagent grade. Cryosolvent solutions were prepared on a v/v basis as described previously (Biringer & Fink, 1982a; Fink & Geeves, 1979). By use of buffer systems based on carboxylate, the temperature effect on pH* was minimized. Absorbance experiments were performed with Cary Model 118 or 219 spectrophotometers; fluorescence measurements were done with a Perkin-Elmer MPF-4 instrument, with excitation at 280 nm and emission at 311.5 nm. Circular dichroism data were collected with a Jasco J-20 or 500A instrument. The temperature of the samples was controlled by thermostating the cells in brass blocks, with circulating ethanol or ethylene glycol–water from Neslab constant-temperature baths.

Methods. The standard protocol used in carrying out the unfolding was as follows: An aliquot of a concentrated stock solution of RNase A, in aqueous or cryosolvent solution, was added to an appropriate volume of the solvent at a temperature below the beginning of the unfolding transition, the final protein concentration being in the 0.05–0.5 mg/mL range. The absence of aggregation under the experimental conditions was confirmed by light scattering experiments on the fluorescence spectrophotometer and by ultracentrifugation (Biringer & Fink, 1982a). The temperature was raised in small increments and the change in spectral signal noted. After equilibrium at each new temperature had been reached, a reading was taken only after the signal had been constant for 10 min. In many cases the sample was stirred with a small magnetic stirring bar. Temperatures were measured by a thermocouple and an Omega or Bailey digital thermometer. For some experiments, a linear temperature programmer (Neslab) was used with a heating rate of 0.3 deg/min and the spectral change monitored continuously. Evaporation of the sample solution was prevented by using tightly stoppered (Teflon) cells and further wrapping Parafilm around the stopper-cell joint. The thermocouple was taped to the side of the cell. In some cases, a silicon septum was used in place of the stopper, and the thermocouple was inserted directly into the cell.

Data Analysis. Data analysis was done with the REDUCE analysis program, developed in part in our laboratory by Dr. Steven Koerber. The fraction of thermally unfolded state (f_U) was obtained as follows: Since the spectral signal of the native and unfolded material frequently exhibited temperature dependence, it was necessary to extrapolate asymptotic lines in these regions to estimate f_U in the transition region. This was done by using expression of the form

$$A_T = (1 - f_U)A_T^N + f_U A_T^U$$

where A_T represents the signal (absorbance, for example) at temperature T, A_T^N represents the signal due to the native state at temperature T, A_T^U represents the signal from the unfolded state at that temperature, and f_U is the fraction unfolded at T. The magnitude of the extinction coefficient change at 286 nm was relatively independent of methanol concentration up to 70% but decreased significantly at higher concentrations.

The data for the unfolding transition were analyzed with the van't Hoff equation, in the form

$$\ln K = a - (\Delta H_0/R)(1/T) + \alpha \ln T \qquad (1)$$

where ΔH_0 is the value of ΔH extrapolated to 0 K and α is the first virial coefficient for ΔC_p. The solid curves in Figures 1, 4, and 5 are based on curve fitting to this equation. The apparent equilibrium constant K was defined as $K = f_U/(1 - f_U)$. Unless otherwise noted, the unfolding was monitored by the change in absorbance at 286 nm.

Results

Effect of Methanol on the Thermal Denaturation Transition. The reversibility of the thermal unfolding at several methanol concentrations up to and including 60% and at different pH* values for 50% methanol was investigated and found to hold. In each case, essentially 100% reversibility was noted. The unfolding transition was determined at increments of 10% methanol (v/v) from 0 to 80%. Figure 1 shows some typical data for the effect of methanol at pH* 3.0 on the unfolding transition as monitored by the absorbance change at 286 nm. At this wavelength, the degree of exposure or burial of tyrosine residues is monitored. In the native protein, two to three of the six tyrosine residues are buried.

The major points to note in this figure are that as the methanol concentration increases the transition moves to lower temperature, as the methanol concentration increases the

[1] Abbreviations: RNase, ribonuclease; NMR, nuclear magnetic resonance; N, native; U, unfolded; pH*, apparent pH of cryosolvent; Gdn·HCl, guanidine hydrochloride; f_N, fraction in native state; f_U, fraction in unfolded state; CD, circular dichroism.

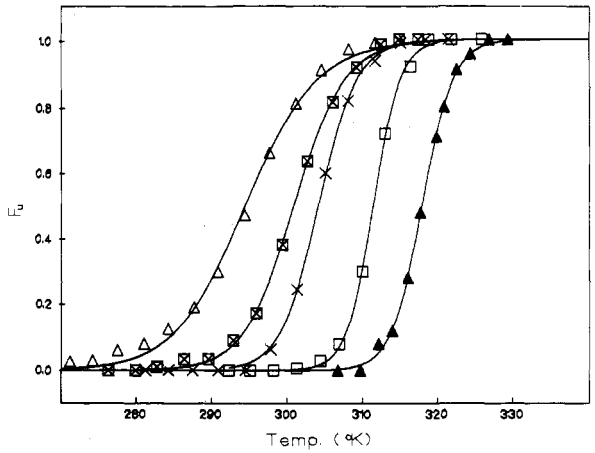

FIGURE 1: Thermal denaturation transition of RNase A as a function of methanol concentration, monitored by absorbance change at 286 nm. The pH* was 3.0. f_U represents the fraction of the unfolded state and was determined as described in the text: aqueous (▲), 20% (□), 40% (×), 50% (⊠), and 70% methanol (△).

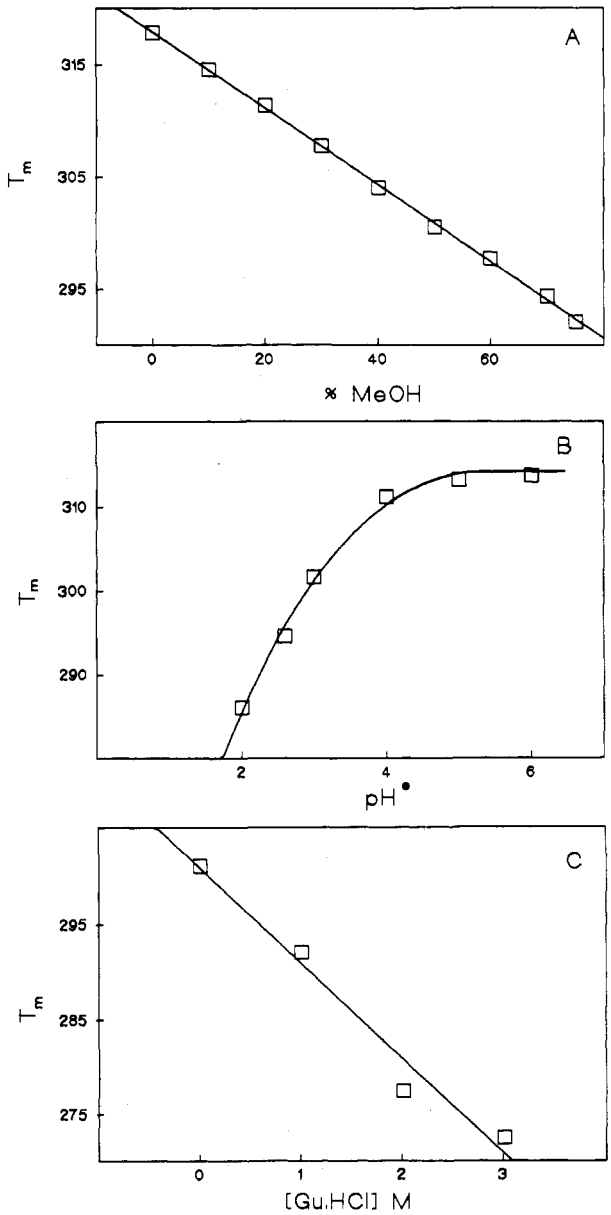

FIGURE 2: Variation of T_m (the temperature at which $f_U = 0.5$) for the unfolding transition of ribonuclease as a function of (A) methanol, (B) pH*, and (C) Gdn·HCl, at pH* 3.0, 50% methanol, unless otherwise noted.

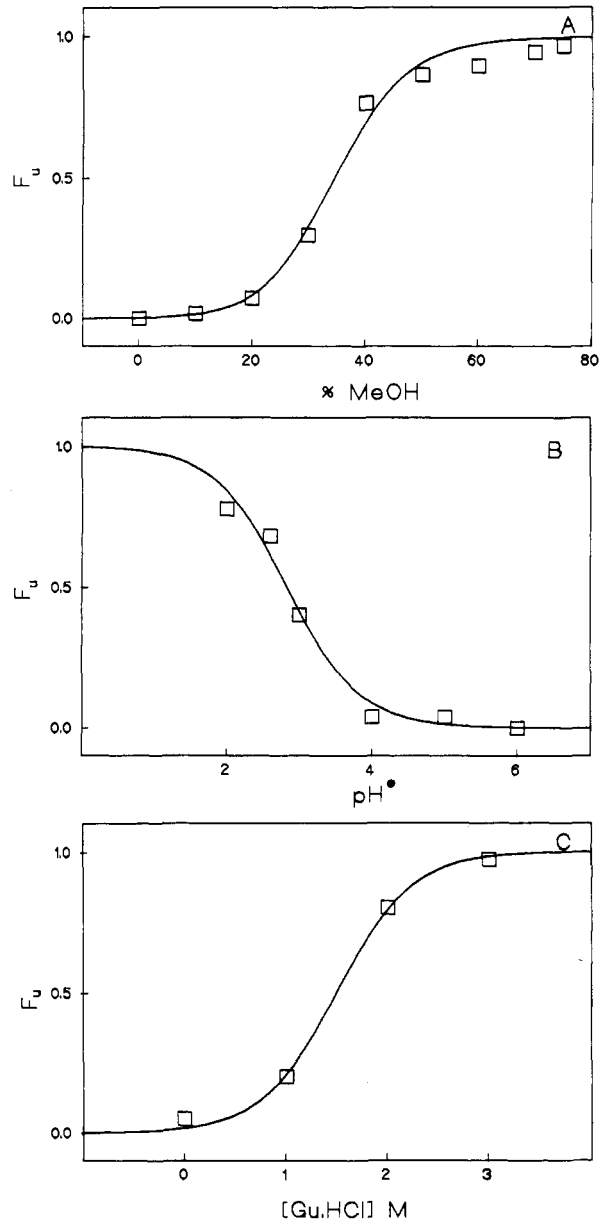

FIGURE 3: Effect of methanol concentration, pH*, and Gdn·HCl (in 50% methanol) on the unfolding transition of ribonuclease at constant temperature. f_U represents the fraction unfolded, at 50% methanol, pH* 3.0, unless otherwise noted. (A) Effect of methanol concentration at 34 °C, (B) effect of pH* at 27 °C, and (C) effect of Gdn·HCl at 14.5 °C.

cooperativity of the transition decreases, and the transition covers a much wider range of temperature. The decreased cooperativity is indicative of populated intermediate states. Figure 2A shows that the T_m ($f_U = 0.5$) decreases in a linear relationship with increasing methanol concentration. The effect of methanol on the isothermal stability of ribonuclease A is shown in Figure 3A, for 34 °C, pH* 3.0. We have chosen 50% methanol, pH* 3.0, as the standard set of conditions for further studies.

Effect of pH on the Thermal Unfolding in Methanol.* The effect of pH* on the unfolding transition in 50% methanol is shown in Figure 4, as monitored by ΔA_{286}. The data for pH* 6.0 are not shown since they were very similar to those at pH* 5.0. The solid lines are theoretical curve fits with eq 1. The transition occurs at increasingly lower temperature as the pH decreases below pH* 6. It is interesting that the transition becomes progressively less cooperative as the pH* decreases, again indicative of populated intermediate states. Plots of pH*

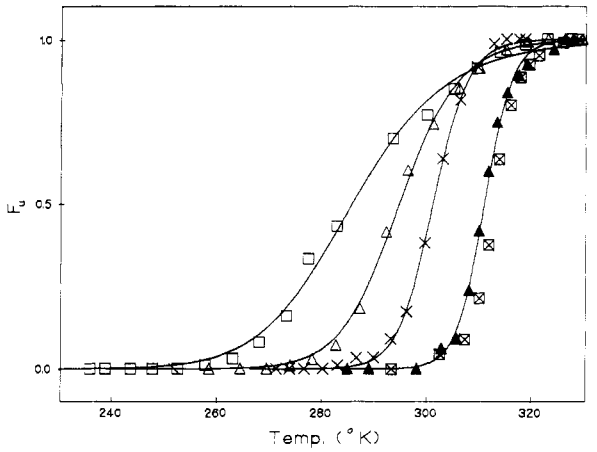

FIGURE 4: Effect of pH* on the thermal unfolding transition for RNase A in 50% methanol. The reaction was monitored with absorbance at 286 nm. f_U represents the fraction in the unfolded state: pH* 2.0 (□), pH* 2.6 (△), pH* 3.0 (×), pH* 4.0 (▲), and pH* 5.0 (⊠).

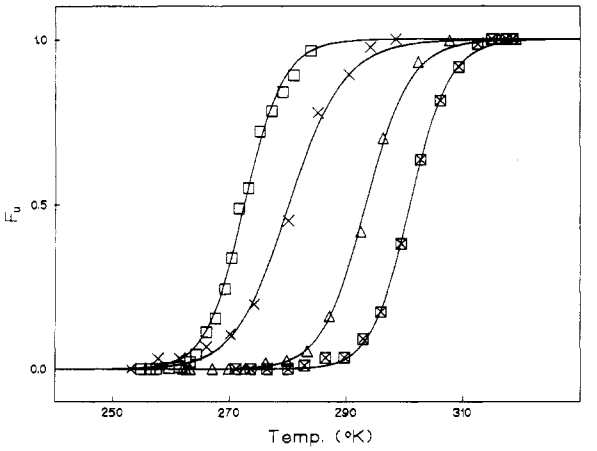

FIGURE 5: Effects of guanidine hydrochloride on the thermal denaturation of RNase A in 50% methanol, pH* 3.0. The transition was monitored by absorbance change at 286 nm: 0 (⊠), 1.0 (△), 2.0 (×), and 3.0 (□) M Gdn·HCl.

vs. T_m (f_U = 0.5) and pH* vs. f_U at constant temperature (27 °C) for 50% methanol are shown in Figures 2B and 3B.

Effect of Guanidinium Chloride on the Transition in Methanol. Guanidinium chloride decreases the stability of the native state leading to lower temperatures for the unfolding transition, as shown in Figure 5, which shows the effect of increasing Gdn·HCl concentration on the reaction in 50% methanol, pH* 3.0, as monitored by changes in absorbance. There is no significant change in the cooperativity of the unfolding transition until 3 M Gdn·HCl is reached, at which point a small increase in cooperativity is noted. The T_m decreases in an essentially linear manner as the guanidine hydrochloride concentration increases (Figure 2C). The variation in f_U as a function of [Gdn·HCl] at constant temperature (14.5 °C) in 50% methanol is shown in Figure 3C.

Unfolding Monitored by Other Techniques. The thermal unfolding in 50% methanol, pH* 3.0, was also monitored with fluorescence and circular dichroism. Some of these data are shown in Figure 6. There is a significant difference in the unfolding transition when monitored by far-UV circular dichroism (θ_{220}), which reflects secondary structure, and by absorbance or fluorescence, which reflects tyrosine exposure and hence tertiary structure. The transition observed by ΔA_{286} coincides with the transition monitored by fluorescence. The circular dichroism results show evidence for a pretransition effect in the −10 to 10 °C region, a much broader transition

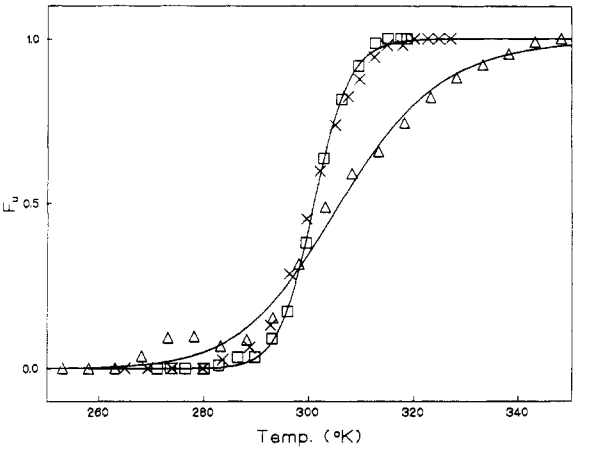

FIGURE 6: Thermal denaturation of RNase A in 50% methanol, pH* 3.0, as monitored by absorbance at 286 nm (□), fluorescence emission (excitation at 280 nm, emission at 311.5 nm) (×), and circular dichroism (ellipticity at 220 nm) (△).

than that seen by absorbance or fluorescence (covering approximately 85 °C), completion of the unfolding transition at 75 °C (compared to 40 °C with absorbance and fluorescence), and that about 40% of the transition occurred at temperatures above that at which no further absorbance or fluorescence change was observed. A more detailed investigation of the unfolding measured by circular dichroism will be reported subsequently (A. L. Fink, B. Lustig, and S. Moore, unpublished results).

Effect of Methanol on Carboxymethylated, Reduced Ribonuclease A. As a method of ascertaining the structure-inducing effect of methanol on ribonuclease under the experimental conditions, we prepared the reduced protein with the thiol groups alkylated to prevent reoxidation. This material was chosen as a model for the unfolded protein, since it has been reported to be relatively devoid of structure, although some studies indicate the presence of significant secondary structure (Takahashi et al., 1977) and possible catalytic activity (Garel, 1978).

Circular dichroism spectra were collected for the alkylated (reduced) and unmodified proteins in 50% methanol, pH* 3.0, at intervals over the temperature range from −15 to 70 °C. The ellipticities at 220 nm are shown as a function of temperature in Figure 7A. For the reduced, alkylated protein, the far-UV CD spectra showed evidence for increasing structure (as inferred from the decrease in θ_{220}) at lower temperatures. However, as shown in Figure 7A, there is a smooth and gradual change in the ellipticity at 220 nm over the total temperature range, with no evidence for a definite transition, unlike the case for the unmodified protein. In addition, the modified protein's spectra at lower temperatures showed little secondary structure and bore little resemblance to those of the unmodified ribonuclease, Figure 7B.

Discussion

There have been a number of previous investigations of the effect of alcohols on the stability of proteins. These include those of Schrier et al. (1965), Brandts and Hunt (1967), and Gerlsma and Stuur (1976) on RNase A and several concerning lysozyme, another small monomeric, globular protein [e.g., Parodi et al. (1973), Gerlsma and Stuur (1974), and Velicelebi and Sturtevant (1979)]. These studies also reported decreases in the transition temperature for unfolding in the presence of alcohols as noted here for methanol and RNase A. This study is the first to use such high concentrations of alcohol; previously the maximal concentrations were no higher than 40% meth-

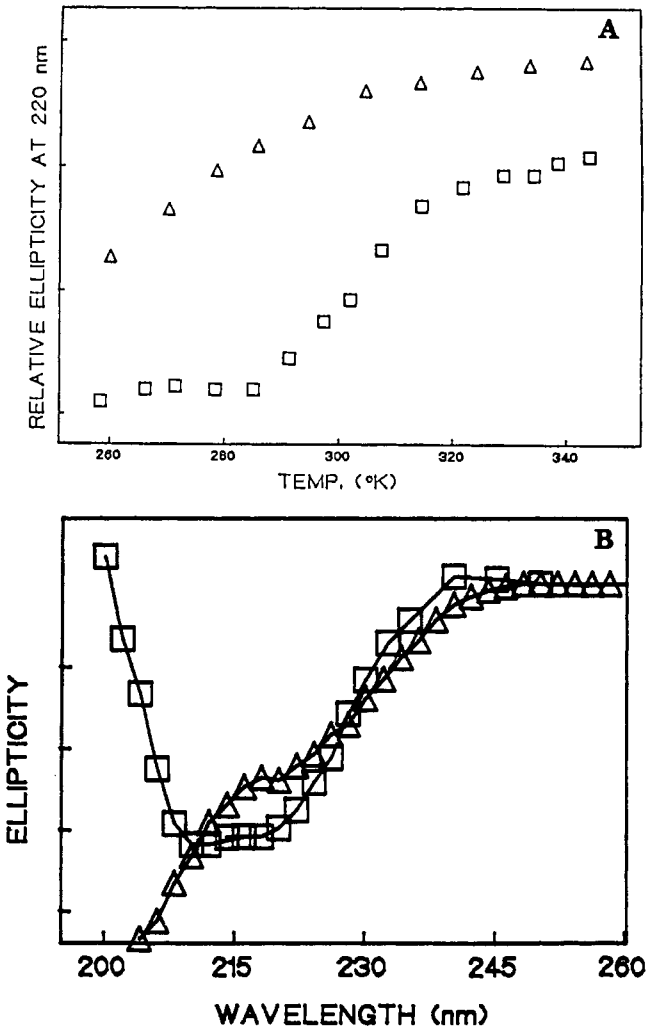

FIGURE 7: Temperature-dependent changes in the molar ellipticity (220 nm) of ribonuclease (□) and alkylated, reduced ribonuclease (△) in 50% methanol, pH* 3.0 (A). (B) shows the spectra of the unmodified protein (□) and alkylated, reduced enzyme (△), both in 50% methanol, pH* 3.0, at 21 °C.

anol, and previous investigations were not carried out at subzero temperatures. The effects of alcohols on unfolding have usually been attributed to favored solvation of the unfolded state in the presence of cosolvent.

The major goal of this investigation was to determine the effects of methanol as a function of concentration, pH*, and [Gdn·HCl] on the position of the unfolding transition of RNase A. This type of information is essential in order to plan experiments to study the kinetics of folding and unfolding in the presence of methanol and at subzero temperatures (R. G. Biringer and A. L. Fink, unpublished results). In addition, we hoped to gain information about the folding process under equilibrium conditions, facilitated by the ability of methanol and low temperatures to stabilize partially folded intermediates.

Using proton NMR, we have previously shown that the structure of *native* RNase A in the presence of high concentrations of methanol under native conditions must be very similar to that in aqueous solution (Biringer & Fink, 1982a). This has also been confirmed by X-ray crystallographic studies, which show that the native conformation of RNase A crystallized from high salt and alcohols is the same (W. A. Gilbert and G. A. Petsko, personal communication).

Effect of Methanol on the Position of the Unfolding Transition. Figures 1, 2A, and 3A show that methanol destabilizes the native state, thus causing the unfolding transition to move to lower temperatures as the methanol concentration increases. This effect stems from the more hydrophobic nature of the cryosolvent compared to that of aqueous solution. Timasheff and co-workers (Pittz & Timasheff, 1978; Gekko & Timasheff, 1981a,b) have shown that the major effect of several organic solvents on proteins is preferential exclusion of the cosolvent from the vicinity of the protein; that is, the protein is preferentially hydrated. In the case of 2-methyl-2,4-pentanediol, Pittz and Timasheff (1978) have suggested that the basis of this phenomenon is electrostatic, involving unfavorable potential interactions between charged groups on the surface of the protein and the relatively hydrophobic cosolvent. We believe that this is a general effect for the monohydric alcohols; that is, the preferential exclusion of the cosolvent reflects the unfavorable interaction between the relatively nonpolar cosolvent and the charged surface of the protein (Fink, 1986). The more hydrophobic cosolvent is also expected to bind preferentially to the unfolded state with its greater hydrophobic surface area, as well as to partially folded intermediate states with exposed nonpolar surfaces.

The transition curves shown in Figures 1, 4, and 5 enable one to determine the temperature at which the protein will be completely native or unfolded as a function of solvent, pH*, and [Gdn·HCl]. For example, the data for the effect of guanidine (Figure 3C) indicate that a 2-fold dilution of a 3 M Gdn·HCl solution in 50% methanol would take one from a situation with predominantly unfolded protein to one in which folded ribonuclease predominated, conditions suitable, for example, for using stopped-flow kinetics experiments to monitor refolding. Such information is necessary in planning experiments to measure the rates of refolding or unfolding.

In terms of folding experiments at subzero temperatures, cryosolvents of 50% methanol can be used as low as −45 °C, and 70% methanol can be used to −70 °C. The solubility of RNase A in methanol cryosolvents is markedly increased below pH* 4.0. Consequently for methods such as NMR and Raman spectroscopy, it will usually be necessary to work at pH* 2–3 in order to have sufficient sample for a reasonable signal to noise ratio. On the other hand, spectrophotometric studies, because of their greater sensitivity, can be carried out at pH* 5 or higher. Taking the pretransition (see below) into consideration, the data indicate that native conditions exist at pH* 3.0 below −15 °C for 50% methanol and below −25 °C for 70% methanol, and below 20 °C for pH* 6.0, 50% methanol.

Circular dichroism (Figure 6) and NMR (Biringer & Fink, 1982b) experiments reveal that some structure is still present after the apparent completion of the transition monitored by ΔA_{286}. Since the burial/exposure of nonpolar Tyr side chains can be taken as a measure of the tertiary structure, this means that some secondary structure remains after loss of the tertiary structure (see below). This observation has important consequences for protein folding studies in general. The intrinsic absorbance and fluorescence of proteins are frequently used as probes of folding, and it is generally assumed that the protein is fully unfolded when the aromatic side chains are fully solvent exposed. As shown here, this may be an erroneous assumption. Clearly circular dichroism (either at both near- and far-UV wavelengths or in the far-UV in conjunction with absorbance or fluorescence) is a more desirable procedure.

The change in shape of the unfolding transition at low pH* (Figure 4), as well as the effect of pH* on the T_m (Figure 2B), suggests that the state of ionization of carboxyl side-chain residues is important in the folding process. The general trend of decreasing T_m with decreasing pH* observed in the presence

of methanol is similar to that seen in aqueous solution (Brandts & Hunt, 1967). The indication that unfolding is different at very low pH* in the presence of methanol is consistent with similar observations in aqueous solution (Labhardt, 1982; Westmoreland & Matthews, 1973; Benz & Roberts, 1975), which suggest that acid-induced denaturation leads to an unfolded conformation different from that at higher pH values.

Guanidine hydrochloride is the classic destabilizing agent for native proteins. The results in Figure 5 indicate that such an effect also occurs in the presence of methanol. In addition, the presence of Gdn·HCl results in the disappearance of the pretransition (see below).

Pretransition Effects. Both proton NMR (Biringer & Fink, 1982b) and circular dichroism data (Figure 6) reveal evidence for a perturbation occurring before the main transition. This pretransition effect was also observed in some of the experiments using A_{286} to monitor the folding process. Similar pretransition effects are observed with aqueous solvent by using X-ray diffraction to monitor structural changes during thermal unfolding of ribonuclease A crystals (Gilbert et al., 1982) and can be seen with aqueous solutions by using absorbance and circular dichroism (Ginsburg & Carroll, 1965). We assume the observed spectral changes corresponding to the pretransition effect reflect either global or local loosening of the tertiary structure, or possibly some localized unfolding or partial unfolding.

Multiple Intermediate States. Evidence for the stabilization of multiple intermediate states by methanol is found in the breadth of the transitions that extend from less than 15 °C for 0% methanol to 45 °C for 70% methanol (pH* 3.0) to >60 °C for 50% methanol, pH* 2.0! A simple quantitative measure of the cooperativity is the ratio of absolute temperatures for $f_U = 0.9$ and $f_U = 0.1$. At pH* 3.0, this ratio changes slightly in going from 0 to 30% methanol and then increases in an essentially linear manner as the methanol concentration increases.

Corroborative support for the presence of partially folded intermediate states is found in an investigation using NMR to monitor the unfolding of RNase A in the presence of methanol (Biringer & Fink, 1982b). In this case, slow- and fast-exchange processes on the NMR time scale indicated the existence of several partially folded states. The following observations from the NMR study of the His-C2 protons are germaine to this investigation. At pH* 3.0, 50% methanol, all the native state was lost by 40 °C and converted into partially folded states. On the basis of calculations of peak area of resonances from the native state, a pretransition region covered the range from −15 to 25 °C, the main transition (as determined by loss of the native state) occurring from 35 to 41 °C. The partially folded states did not completely disappear into fully unfolded material until 75 °C.

There are substantial similarities, therefore, between the unfolding monitored by NMR and the unfolding monitored by circular dichroism (Figure 6). Both probes reveal the pretransition in the −15 to 25 °C region and the lack of complete unfolding until 75 °C. The transition monitored by absorbance at 286 nm corresponds closely to the NMR transition observed by loss of the native state (but not loss of the partially folded states). Of particular note is the fact that after the completion of unfolding as judged by the lack of further changes in absorbance at 286 nm, the NMR spectra (and circular dichroism data) indicate the presence of partially folded species existing for another 35 °C. Our interpretation of these observations, elaborated on below, is that the initial stages of unfolding involve *predominantly* the loss of tertiary structure and hence result in exposure of all the tyrosine residues to solvent. This accounts for the apparent complete unfolding as judged by ΔA_{286}. The NMR and far-UV circular dichroism signals however are sensitive to the residual, predominantly secondary, structure that remains at this stage of unfolding and is only lost at considerably higher temperatures.

It is thus the presence of the relatively more hydrophobic methanol that can stabilize partially folded intermediate states with exposed nonpolar groups that is responsible for the observed stabilization of folding intermediates. The fact that low concentrations of Gdn·HCl do not increase the cooperativity of the unfolding transition in methanol implies that the partially folded intermediates are still present and thus relatively resistant to destabilization under these conditions.

Tertiary Structure Is Lost before Secondary Structure. The lack of coincidence of the θ_{220} curve with that of absorbance and fluorescence (Figure 6) indicates that the loss of tertiary structure is not coincident with the loss of secondary structure when RNase A is unfolded in the presence of 50% methanol. Combining this observation with those from NMR and reported above leads one to a minimum model of the sort shown in Scheme I to account for the unfolding of ribonuclease A in the presence of high methanol concentrations. In this scheme, N'_1 represents the species present at completion of the pretransition, I_s corresponds to the species that exists at the completion of the transition as monitored by absorbance and has lost its tertiary structure, and U* represents the final *thermally* unfolded state, which may still retain residual structure under some conditions (Labhardt, 1982). NMR observations indicate that the presence of methanol aids in the destabilization of this residual structure, since NMR results show that the protein appears fully unfolded in 50% methanol at 75 °C (Biringer and Fink, 1982b), in contrast to the case in aqueous solution.

Scheme I

$$N \leftrightarrow N'_1 \leftrightarrow I_s \leftrightarrow U^*$$

These observations are consistent with a model of protein folding in which the initial stage is the formation of local regions of metastable units of structure, including secondary structural units. These short-lived structural units, which are predominantly stabilized by hydrogen bonding, flicker back and forth with the unfolded state until their interactions result in the formation of more stable, larger units of structure, probably through both hydrogen-bonding and hydrophobic interactions. These units, in turn, pack with each other, predominantly through hydrophobic interactions due to the presence of exposed nonpolar residues on their surfaces. Eventually sufficiently stable structural regions will form to result in the existence of discrete intermediate states, which will then lead to a loosely packed, tertiary structure that undergoes final conformational rearrangements and solvent exclusion to give the native state. It is known that the internal surfaces of units of secondary structure in proteins are predominantly hydrophobic in nature [e.g., Chothia et al. (1981) and Eisenberg et al. (1986)].

Since ribonuclease is effectively a single domain protein, a multiplicity of states in its unfolding cannot be attributed to independent unfolding of domains. Consequently, the evidence for more than two states (N and U) indicates that there must indeed be partially folded states present. Whether these are directly on the folding pathway or result from initial unfolding followed by subsequent refolding to the observed partially folded state(s) (Scheme II) is more difficult to answer unambiguously.

Scheme II

$$N \leftrightarrow U \leftrightarrow I$$

The tendency of alcohols to induce helical structure in proteins [Tanford (1968) and references cited therein] means that Scheme II is a real possibility. The effect of methanol and temperature on the structure of carboxymethylated, reduced RNase was investigated as a model system, since in aqueous solution this derivative possesses relatively little structure compared to that of the unmodified protein (Takahashi et al., 1977). Figure 7A shows that there is no discrete transition when methanolic solutions of the modified protein are heated. A smooth change in ellipticity is observed that presumably reflects loss of some nonrandom (local) structure. At temperatures where the unmodified protein exhibits a nativelike circular dichroism spectrum, the derivative shows relatively little structure (and is similar to the spectrum in aqueous solution). Thus, although the presence of methanol induces some additional structure in the reduced, alkylated protein as compared to aqueous solution, the amount is small and is temperature-dependent. Consequently, the results for the effect of methanol on unmodified ribonuclease are consistent with Scheme I rather than Scheme II.

Although there have been previous reports of multiphasic unfolding transitions (normally due to independent unfolding/refolding domains), we believe that the results reported here are the first unambiguous case for the predominant loss of tertiary structure prior to loss of secondary structure. It is the stabilizing effect of the relatively hydrophobic methanol on the exposed nonpolar residues of the partially folded states that permits their observation. If the process of folding is the opposite of unfolding, then we predict that in folding secondary structure will be formed prior to tertiary structure.

Several studies of the free energy of denaturation as a function of temperature have suggested that a maximum occurs in the ΔG_d vs. temperature plot, i.e., that unfolding should occur at both high and low temperatures (Pace & Tanford, 1968; Schellman & Hawkes, 1980; Ghélis & Yon, 1982). For RNase A Brandts and Hunt (1967) have shown that the extrapolated maxima of these curves occur around 0 °C, as confirmed in this study. Our data predict that ribonuclease A should be 50% unfolded at −45 °C at pH* 3, in 70% methanol, due to the cold denaturation. However, in experiments measuring the catalytic activity toward CpA at pH* 3.0 in 70% methanol we observe substantial activity even at −70 °C (A. L. Fink, D. Kar, and R. Kotin, unpublished results). Preliminary investigations to detect directly the anticipated "cold denaturation" in 70% methanol, pH* 3.0, at −45 °C, have failed to reveal any evidence for unfolding. It is possible that the rate of unfolding under these conditions is sufficiently slow that the native state is effectively stabilized.

It is not clear whether the effects of methanol on the folding of ribonuclease A are typical of all globular proteins, since Velicelebi and Sturtevant (1979) have found that the ratio of the calorimetric to van't Hoff enthalpies is effectively unity for lysozyme unfolding to at least 25% (v/v) methanol, indicating apparent adherence to a two-state system.

ACKNOWLEDGMENTS

We thank C. R. Matthews and D. Goldenberg for their suggestion to use alkylated, reduced ribonuclease A as a model for unfolded protein.

Registry No. Methanol, 67-56-1; ribonuclease A, 9001-99-4; guanidine hydrochloride, 50-01-1.

REFERENCES

Benz, F. W., & Roberts, G. C. K. (1975) *J. Mol. Biol. 91*, 345–365.
Biringer, R. G., & Fink, A. L. (1982a) *Biochemistry 21*, 4748–4755.
Biringer, R. G., & Fink, A. L. (1982b) *J. Mol. Biol. 160*, 87–116.
Brandts, J. F., & Hunt, L. (1967) *J. Am. Chem. Soc. 89*, 4826–4838.
Chothia, C., Levitt, M., & Richardson, D. (1981) *J. Mol. Biol. 145*, 215–250.
Eisenberg, D., Wilcox, W., & McLachan, A. D. (1986) *J. Cell. Biochem. 31*, 11–17.
Fink, A. L. (1986) *Cryobiology 23*, 28–37.
Fink, A. L., & Geeves, M. A. (1979) *Methods Enzymol. 63*, 336–370.
Garel, J.-R. (1978) *J. Mol. Biol. 118*, 331–345.
Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4667–4676.
Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4677–4686.
Gerlsma, S. Y., & Stuur, E. R. (1974) *Int. J. Pept. Protein Res. 6*, 65–72.
Gerlsma, S. Y., & Stuur, E. R. (1976) *Int. J. Pept. Protein Res. 8*, 3–12.
Ghélis, C., & Yon, J. (1982) *Protein Folding*, Academic, New York.
Gilbert, W. A., Lord, R. C., Petsko, G. A., & Thamann, T. J. (1982) *J. Raman Spectrosc. 12*, 173–179.
Ginsburg, A., & Carroll, W. R. (1965) *Biochemistry 4*, 2159–2174.
Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.
Labhardt, A. M. (1982) *J. Mol. Biol. 157*, 331–355.
Pace, C. N., & Tanford, C. (1968) *Biochemistry 7*, 198–208.
Parodi, R. M., Bianchi, E., & Ciferri, A. (1973) *J. Biol. Chem. 218*, 4047–4051.
Pfiel, W., & Privalov, P. L. (1976) *Biophys. Chem. 4*, 41–50.
Pittz, E. P., & Timasheff, S. N. (1978) *Biochemistry 17*, 615–622.
Schellman, J. A., & Hawkes, R. B. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 331–343, Elsevier/North-Holland, New York.
Schrier, E. E., Ingwall, R. T., & Scheraga, H. A. (1965) *J. Phys. Chem. 69*, 298–303.
Takahashi, S., Kontani, T., Yoneda, M., & Ooi, T. (1977) *J. Biochem. (Tokyo) 82*, 1127–1133.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121.
Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180–1186.
Westmoreland, D. G., & Matthews, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 914–918.
White, F. H., Jr. (1967) *Methods Enzymol. 11*, 481–484.