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Folding of Ribonuclease A from a Partially Disordered Conformation. Kinetic Study under Folding Conditions†

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ABSTRACT: Bovine pancreatic ribonuclease A (RNase) was partially disordered with 3.5 M $LiClO_4$ (pH 3.0). The conformation of this partially disordered material was studied by circular dichroism and Raman spectroscopy. Although the partially disordered protein appears to have a lower β-structure content and disordered tyrosyl side chains, compared to native RNase, it seems to retain some ordered backbone structure that is suggested to be α helix. The kinetics of folding of $LiClO_4$-denatured RNase was studied by means of absorption and circular dichroism measurements. For comparison, the kinetics of folding of urea-denatured RNase (which is completely devoid of ordered structure) was examined with the same techniques. Since the kinetics of folding of both denatured species are found to be similar, it appears that the ordered structure present in $LiClO_4$-denatured RNase plays no role in determining the folding pathway. Also, the change in the circular dichroism at 220 nm showed that some of the ordered structure in $LiClO_4$-denatured RNase becomes disordered in the early stages of folding. This implies that all ordered structures in RNase are not equivalent in their influence on the folding pathway; some can play an essential role and some may not.

Konishi et al. (1982b) proposed two types of pathways for protein folding. One is designated as a growth-type pathway in which the nucleation sites are folded in the rate-limiting step and other parts of the polypeptide chain fold around the nucleation sites. In this pathway, native interactions play a significant role in influencing folding. In the second, a rearrangement-type pathway, some nonnative interactions are essential for folding, and the disruption or rearrangement of these interactions to native ones constitutes the rate-limiting step. In this paper, we examine the role or effect of ordered structure in partially disordered RNase[1] (which is disordered by a high concentration of $LiClO_4$) on the proposed folding pathways.

Konishi et al. (1982a) have pointed out that, in studies of the folding pathways of proteins, analysis of the preequilibrium state (as, for example, in the regeneration of RNase) gives

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[1] Abbreviations: RNase, bovine pancreatic ribonuclease A; N-RNase, native RNase; D(urea)-RNase, RNase denatured by urea (the denaturant is enclosed in parentheses); I(urea)-RNase, intermediate in the folding/unfolding of RNase by urea; A_t and A_∞, absorbance at time t and infinite time, respectively, in the kinetic experiments; CD, circular dichroism; UV, ultraviolet; Gdn·HCl, guanidine hydrochloride; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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information about the relative stabilities of the "Intermediates"[2] that exist prior to the rate-limiting steps[3] but not about the pathways; kinetic data are required to obtain information about the pathways, especially about the rate-limiting steps. Since this paper is concerned with the role that ordered structures play in the rate-limiting steps in protein folding, a kinetic method is adopted.

Brandts et al. (1975) have proposed that the slow interconversion between two types of denatured conformations[4] reflects the cis–trans isomerization of proline residues. This hypothesis has received considerable support by other investigators (Brandts et al., 1977; Schmid & Baldwin, 1978; Lin & Brandts, 1978; Stellwagen, 1979; Garel, 1980; McPhie, 1980, 1982; Kato et al., 1982). Recent studies of the folding of RNase (Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981), however, indicate that proline isomerization (Pro-93 in the case of RNase) occurs during the conversion from a mostly folded "Intermediate*"[2] (Konishi et al., 1982b) to fully refolded RNase. Thus, the proline-isomerization hypothesis does not answer the fundamental question as to how a protein is folded up into its native conformation.

One approach to this question would involve analysis of the conformations of the intermediates. Such an analysis was carried out for the intermediates on the regeneration pathway of bovine pancreatic trypsin inhibitor by immunochemical (Creighton et al., 1978) and UV difference–absorption spectroscopic (Kosen et al., 1980) techniques. The results indicated that the conformations of the intermediates become more compact after disulfide bonds are formed; those of the "Intermediates" are predominantly denatured whereas that of a trapped "Intermediate*" is nativelike, indicating that most of the folding to the native conformation occurs in the rate-limiting step. A similar phenomenon was observed for the intermediates in the regeneration of RNase from the reduced protein (Creighton, 1979; Konishi & Scheraga, 1980a,b; Galat et al., 1981), even though a small amout of nativelike local folding was found in reduced RNase (Chavez & Scheraga, 1980). In kinetic studies of the folding of RNase from D-(Gdn·HCl)-RNase,[1] Schmid & Baldwin (1979) detected some ordered structure in the "Intermediates" by means of amide proton exchange experiments. As pointed out by Konishi et al. (1982a), a comparison of the conformations of the "Intermediates" and the "Intermediates*" would provide important information about the structures that are folded in the rate-limiting step(s).

Another approach to the above question would involve a folding experiment that started from a partially disordered species. Since the nucleation site(s) is (are) folded in the rate-limiting step(s) in the folding pathways, the partially disordered species can be classified as an "Intermediate*" or an "Intermediate" depending on whether it does or does not contain the nucleation site(s). If the ordered structure in this starting material does encompass the nucleation site(s) for proper folding, then this species would be classified as an "Intermediate*", and the folding from such an "Intermediate*" would normally be very rapid because it would already have passed through the rate-limiting step (Konishi et al., 1982b). An example of such a very rapid folding transient "Intermediate*" that could be detected experimentally might be the one observed by Hagerman & Baldwin (1976) and Hagerman et al. (1979) in the folding of RNase. On the other hand, if the ordered structure in the starting material does not encompass the proper nucleation site(s), then this species would be classified as an "Intermediate". Since the folding from such an "Intermediate" would require passage through the rate-limiting step, the process would be slower than the one that started from an "Intermediate*".

Many reports of ordered structure in denatured proteins and in folding intermediates have appeared. Although conformational intermediates have been detected in equilibrium transitions in proteins (Burgess & Scheraga, 1975; Baldwin & Creighton, 1980), their concentrations are usually too low for them to be used as starting species in the folding experiments indicated in the previous paragraph; special methods, such as a double-jump technique, would be required to accumulate them (Hagerman et al., 1979). On the other hand, under some denaturing conditions, some ordered structures are sometimes observed. For example, CD spectra (Saxena & Wetlaufer, 1970; Schaffer, 1975; Takahashi et al., 1977) and immunochemical data (Chavez & Scheraga, 1980) of reduced RNase and lysozyme indicate the presence of some ordered structures; however, the large increase in entropy accompanying the reduction of the disulfide bonds (Konishi et al., 1981, 1982a) tends to disrupt most of the ordered structure. On the other hand, when the disulfide bonds are not disrupted, as, for example, in thermally unfolded RNase, residual local structures are observable by various techniques (Westmoreland & Matthews, 1973; Chen & Lord, 1976; Matheson & Scheraga, 1979a; Matthews & Froebe, 1981). These local structures might arise from the tendencies of hydrophobic and hydrophilic residues to lie inside and outside of the protein, respectively, when the solvent is water, with a maximization of the strength of hydrophobic interactions at about 60 °C (Scheraga, 1963). Acetic acid greatly decreases the amplitude of the aromatic CD spectrum of tyrosine but affects the backbone CD spectrum of native RNase only slightly (Cann, 1971); this indicates that D(acetic acid)-RNase[1] has some ordered backbone structure but disordered tyrosine side-chain conformations. Similarly, ordered structures have been observed for α-lactalbumin denatured by lowering the pH [D(pH)-α-lactalbumin][1] (Kuwajima et al., 1976); the CD spectrum of the backbone was analyzed in terms of contributions from α helix, β structure, and random coil, with the suggestion that D(pH)-α-lactalbumin appears to have a higher α-helix but a lower β-structure content than the native protein (Kuwajima, 1977; Contaxis & Bigelow, 1981). Neutral salts can act similarly to produce denatured proteins with some ordered structure (von Hippel & Schleich, 1969); many different kinds of neutral salts have been found to denature RNase (von Hippel & Wong, 1965; Ahmad & Bigelow, 1979), and the CD spectra of denatured RNase indicated that $LiClO_4$ was the most effective in disordering the tyrosine side chains without inducing much change in the backbone structure.

[2] "Intermediates" and "Intermediates*" represent the intermediates prior to and after, respectively, the rate-limiting steps[3] in the pathways for the folding of RNase A. "Intermediates" also include the denatured protein because it is situated prior to the rate-limiting step in the pathway.

[3] In this paper, we use the definition of a "rate-limiting step" given by Konishi et al. (1982b), viz., the slowest step in the conformational folding pathway of a protein. However, this does not include slow steps from a mostly folded species to the native protein; these are defined as slow steps *after* the (slow) rate-limiting steps, because we are interested in how the entire protein molecule is folded up and not in the local conformational changes that occur when a mostly folded species is converted to the native protein.

[4] In this paper, we define the denatured conformation of a protein as one in which the *entire* (not simply the local) conformation is *nonnative* and induced by a denaturant such as high temperature, high pressure, extremes of pH, urea, Gdn·HCl, neutral salts, organic solvents, etc. The nonnative conformation is not necessarily disordered. The details are considered under Discussion.

This paper is devoted to an analysis of the role that ordered structures in denatured RNase play in the kinetics of folding (and in the folding pathways) of this protein. In order to retain some ordered structure in the denatured RNase, we used $LiClO_4$ as the denaturant.

Experimental Procedures

(*A*) *Materials.* Bovine pancreatic ribonuclease A (Sigma Chemical Co., type IIA) was purified on a carboxymethylcellulose column (Taborsky, 1959). Ultrapure urea (Schwarz/Mann) and anhydrous lithium perchlorate (Alfa, 99.5%) were used without further purification. Less than 50 ppm of cyanate was detected by a colorimetric test (Werner, 1923) in 8 M urea solutions at pH 3.

The concentrations of urea solutions were checked by refractive index measurements at 20 °C (Warren & Gordon, 1966). Lithium perchlorate ($LiClO_4$) was weighed accurately in a Manostat drybox under a dry N_2 atmosphere for preparation of solutions.

Sodium cytidine cyclic 2′,3′-phosphate (C>P) and Tris base were purchased from Sigma Chemical Co. Carboxymethylcellulose (Whatman Ltd., CM-52) was used for column chromatography to purify RNase. All solutions were buffered with 50 mM Gly–HCl buffer at pH 3.0 and were routinely clarified with filters (0.45 μm) purchased from Millipore Corp.

A Radiometer Model PHM84 pH meter with a Radiometer glass combination electrode was used for all pH measurements. Salt effects on electrode–liquid junction potential and water activity are known to give a pH error of less than 0.15 at low pH (Bates, 1954; Donovan et al., 1960).

(*B*) *Protein Concentration.* Protein concentrations were determined by Kjeldahl nitrogen analysis (Lang, 1958; Noel & Hambleton, 1976) or by spectrophotometric methods using $\epsilon_{277.5} = 9800\ M^{-1}\ cm^{-1}$ for N-RNase (Sela & Anfinsen, 1957). For D($LiClO_4$)- and D(urea)-RNase in 3.5 M $LiClO_4$ (pH 3) and in 8 M urea (pH 3), respectively, the extinction coefficient was determined as follows: Three identical aliquots of a concentrated RNase solution of known concentration were diluted to the same final volumes to obtain D($LiClO_4$)-, D-(urea)-, and N-RNase solutions, respectively, of identical protein concentrations. Using $\epsilon_{277.5} = 9800\ M^{-1}\ cm^{-1}$ for N-RNase, we obtained a value of $\epsilon_{275} = 9000\ M^{-1}\ cm^{-1}$ for both D($LiClO_4$)- and D(urea)-RNase.

The absorption measurements were made on a Cary Model 14 spectrophotometer, modified by V. G. Davenport to decrease the full-scale optical density by a factor of 10 (i.e., the lowest optical density was altered from a full scale of 0.1 to one of 0.01). The data were digitized automatically with an analog-to-digital converter connected to the spectrophotometer and then input directly to a computer.

(*C*) *Molecular Weight Measurement.* The weight-average molecular weight of RNase in 3.5 M $LiClO_4$ was determined by conventional sedimentation equilibrium with a Spinco Model E ultracentrifuge. The concentration was determined by using the absorption optics system of the ultracentrifuge and an electronic scanning system designed and constructed by V. G. Davenport (Scheule et al., 1976).

(*D*) *Raman Measurements.* A spectrometer that was described previously (Maxfield & Scheraga, 1977; Scheule et al., 1977) was used to obtain Raman spectra. The spectra were recorded and stored in digital form and plotted on a Hewlett-Packard 7220S plotter. The 488.0-nm line of a Coherent CR3 argon ion laser at a power of 150–200 mW was focused on a 3 × 3 × 40 mm fluorescence cell (Precision Cells, Inc.). The cell contained 150–300 μL of solution that was maintained at 4 °C with a brass block cooled by a Forma-Temp Jr. bath and circulator. The protein concentration was 10% (w/v). The temperature was measured by a calibrated, dipping thermistor. The instrumental resolution was 7 cm^{-1} because the protein peaks were of low intensity. Peak frequencies were calibrated with the plasma lines of the argon laser (Loader, 1970; Craig & Levin, 1979). All spectra were reproduced at least twice and then averaged with a computer. The estimated accuracy of sharp bands is ±2 cm^{-1}. Solvent base lines were subtracted with a computer. No change in enzymatic activity of RNase against C>P [measured spectrophotometrically by the method of Crook et al. (1960)] was detected after 12 and 24 h of laser irradiation.

(*E*) *Circular Dichroism Measurements.* Circular dichroism (CD) measurements were made with a CD attachment, Model 6001, to a Cary Model 60 spectropolarimeter. Quartz cells with 0.3- and 1-cm path lengths and with water jackets were used. A Haake Type F circulating bath was used to keep the temperature constant within ±0.1 °C. The temperature was measured with a calibrated thermistor probe. The protein concentration was (2.0–9.0) × 10^{-5} M. The instrument was calibrated with *d*-10-camphorsulfonic acid (Adler et al., 1973) and D-pantolactone (Tuzimura et al., 1977).

Kinetic measurements were made at 275 and 220 nm with an instrumental response time of 1 s at 4 °C. In a typical experiment, 100 μL of a 3.0% (w/v) RNase solution in 3.5 M $LiClO_4$ (pH 3.0) was added to 4.0 mL of 0.51 M urea solution (pH 3.0) in a large test tube with a Gilson Model P200 micropipetter. The final concentrations[5] of the reagents were [RNase] 5.3 × 10^{-5} M, [$LiClO_4$] 0.085 M, and [urea] 0.50 M. After the solution was mixed quickly, the CD cell was filled immediately with the solution and $[\theta]_{275}$ or $[\theta]_{220}$ recorded. The solutions, test tubes, cell, and pipet tips were chilled previously for at least 30 min at 4 °C. Because of the limiting precision of the signal-to-noise factor, the kinetic parameters obtained from the CD measurements are considered to be only approximate.

(*F*) *Difference Absorbance Measurements.* The same modified Cary Model 14 spectrophotometer was used with two matched 1-cm silica cells for difference absorbance measurements. Thermal transition studies of RNase were carried out by keeping the reference protein solution at 20 °C and changing the temperature of a brass block in contact with the matched cell with a Forma-Temp Jr. bath and circulator with an accuracy of ±0.1 °C. The temperature was measured with a calibrated dipping thermistor.

Kinetic experiments were carried out by manual mixing of the solutions, as in the case of the CD measurements. In a typical experiment, 60 μL of a 2.5% (w/v) RNase solution in 3.5 M $LiClO_4$ (pH 3.0) was added with a Gilson Model P200 micropipetter to 2.4 mL of 0.51 M urea (pH 3.0) in a cell in the spectrophotometer that (together with the solutions and pipet tips) had been thermally equilibrated for at least 30 min at 4 °C. Because the dilution of urea was very slight, no significant change of temperature occurred despite the high heat of dilution. The final concentrations[5] of the reagents were [RNase] 4.5 × 10^{-5} M, [$LiClO_4$] 0.085 M, and [urea] 0.50 M. Immediately after stirring the solution quickly, we recorded the time dependence of the absorbance change. Measurements were made until no further change in absorbance was detectable (typically ca. 6 times the larger relaxation time). After dilution, the pH of the solution was checked and found to be 3.0.

[5] These are "folding" conditions. The final value of [urea] was 0.50 M, rather than 0, for later comparison with runs in which the urea concentration was changed from a high value to a low one, viz., 0.50 M.

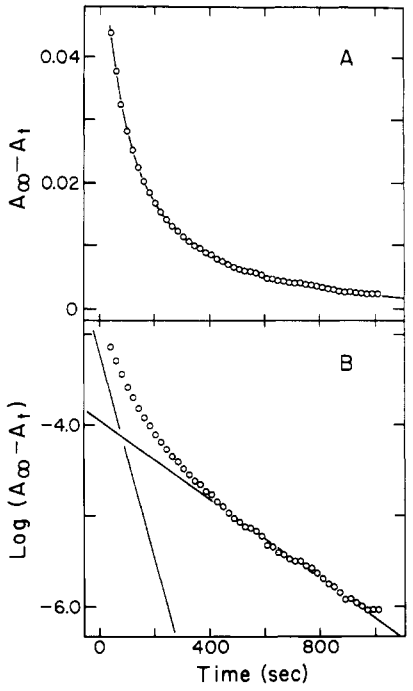

FIGURE 1: Time dependence of (A) difference absorbance and (B) logarithm of difference absorbance at 287 nm of RNase after change of solvent from 3.5 M $LiClO_4$ (pH 3.0) to 0.085 M $LiClO_4$ and 0.50 M urea (pH 3.0) at 4 °C. The concentration of RNase decreased from 1.83×10^{-3} to 4.46×10^{-5} M with the change in solvent. The absorbance increase is represented by the experimental points. The solid lines show (A) the computed best fit to the data and (B) the two separate exponential terms in the rate equation (1).

(*G*) *Stopped-Flow Measurements.* In order to examine conformational changes that occur faster than could be observed by the manual mixing technique used for the absorbance measurements described in section F, we carried out stopped-flow measurements with a Durrum-Gibson stopped-flow apparatus at room temperature. In a typical experiment, a solution of 7.5×10^{-4} M RNase in 3.5 M $LiClO_4$ (pH 3.0) was placed in the smaller syringe, and 50 mM Gly–HCl buffer (pH 3.0) was placed in the larger syringe. The solutions from both syringes were rapidly introduced into the mixing chamber with a dilution factor of 7.8 for both RNase and $LiClO_4$. Thus, the final concentrations of the reagents, after the solvent jump, were [RNase] 9.6×10^{-5} M and [$LiClO_4$] 0.45 M. Transmittance at 235 or 287 nm through a 2-cm cell was measured with a photomultiplier tube. Dilution trials of concentrated denaturant solutions without protein showed small mixing artifacts in absorbance scans, e.g., sharp peaks due to the large change in refractive index upon mixing. These artifacts were minimized by increasing the protein concentration above 0.5 absorbance unit.

(*H*) *Analysis of Kinetic Data for Folding/Unfolding.* Since the observed kinetic data for the time dependence of absorption changes showed two distinct phases (see Figure 1), the kinetics for the folding/unfolding process were analyzed with the following empirical equation involving two phases with apparent time constants τ_{1s} and τ_{2s} ($\tau_{1s} < \tau_{2s}$):

$$A_\infty - A_t = A_{1s}e^{-t/\tau_{1s}} + A_{2s}e^{-t/\tau_{2s}} \quad (1)$$

$$f_2 = A_{2s}/(A_{1s} + A_{2s}) \quad (2)$$

where A_∞ and A_t are the relative absorbances at infinite time (usually ca. 6 times the larger relaxation time) and t min, respectively, after the change of solvent. A_{1s} and A_{2s} are the amplitudes of the absorbance changes of the two phases, and f_2 is the relative amplitude associated with the time constant

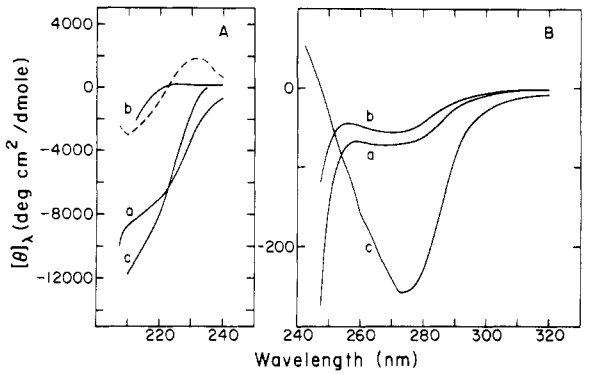

FIGURE 2: Circular dichroism spectra for (a) D($LiClO_4$)-RNase in 3.5 M $LiClO_4$ (pH 3.0), (b) D(urea)-RNase in 8 M urea (pH 3.0), and (c) N-RNase in 50 mM Gly–HCl (pH 3.0) at 4 °C in (A) the far-UV and (B) the near-UV regions. The concentrations of RNase were $(3.1–8.9) \times 10^{-5}$ M. The dashed line represents the difference CD spectrum between D($LiClO_4$)- and N-RNase.

τ_{2s}. The subscript s is used to indicate that both phases are slow, compared to the faster process that is complete within the manual mixing time (Baldwin & Creighton, 1980). The data were fit with a computer program that treated the error as a quadratic function and varied the adjustable parameters A_{1s}, A_{2s}, τ_{1s}, and τ_{2s}. The best fit was determined by minimizing the error and its first and second derivatives (Cornish-Bowden & Koshland, 1970; Wharton et al., 1974). Examples of the experimental data and the calculated curves are shown in Figure 1. The kinetic data were reproduced at least twice, and average errors were 3, 10, and 7% for τ_{1s}, τ_{2s}, and f_2, respectively.

Results

(*A*) *Characterization of D($LiClO_4$)-RNase.* The molecular weight of D($LiClO_4$)-RNase in 3.5 M $LiClO_4$ at pH 3.0 and 25 °C was found to be 15 300 (compared to 13 680 for native RNase) in the concentration range of $(8–30) \times 10^{-5}$ M. Recognizing that this value may include a contribution from some bound $LiClO_4$, this result indicates that D($LiClO_4$)-RNase is not significantly aggregated.

The CD data for D($LiClO_4$)-RNase are shown in Figure 2, where they are compared with those for N-RNase and D(urea)-RNase, the latter being a good reference for a disordered denatured conformation (Tanford, 1968, 1970). The similar values of $[\theta]_{275}$ for D($LiClO_4$)-RNase and D(urea)-RNase demonstrate that the tyrosine side chains of both species are disordered. The difference CD spectrum between D($LiClO_4$)- and N-RNase in the range of 200–240 nm is characteristic of a β structure (Chen et al., 1972, 1974), indicating that $LiClO_4$ disrupts some of the β structure of N-RNase. On the other hand, the fact that the CD spectrum in the range of 200–240 nm for D($LiClO_4$)-RNase is similar to that of N-RNase rather than to that of D(urea)-RNase indicates that, despite the loss of some β structure, D($LiClO_4$)-RNase contains some remaining ordered backbone structure.

Raman spectra provide additional data for comparing the three conformational states under the conditions of Figure 2. Even though urea and $LiClO_4$ have intense Raman peaks at 534, 601, 1008, 1167, 1478, 1604, and 1680 cm^{-1} and 462, 628, 935, and 1102 cm^{-1}, respectively (Figure 3) (Otvos & Edsall, 1939; Kohlrausch, 1938), subtraction of the solvent spectra from those of Figure 3 gives spectra that are characteristic of native and denatured RNase (Figure 4) (Lord & Yu, 1970). In proteins, the doublet at ~850 and ~830 cm^{-1} is due to Fermi resonance interactions between the normal

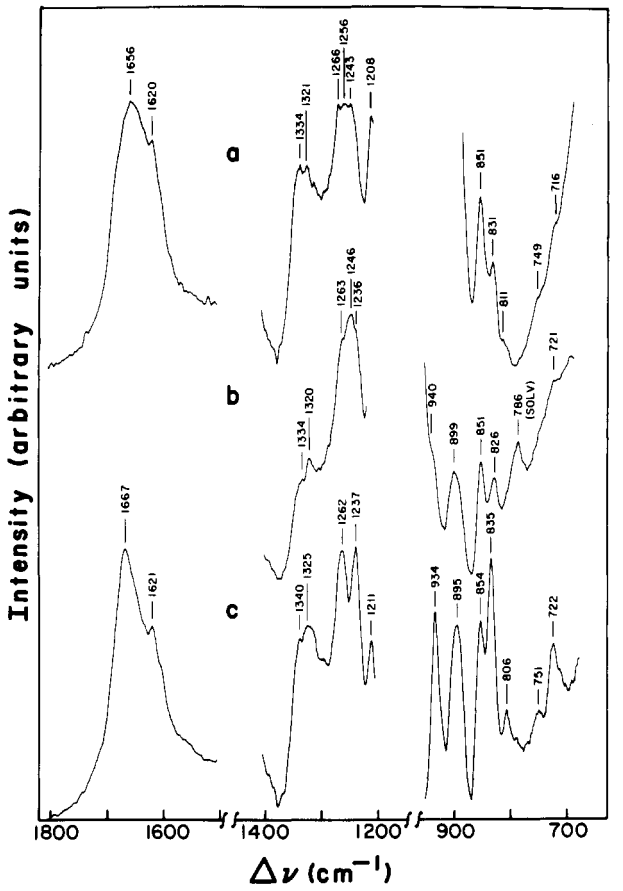

FIGURE 3: Raman spectra for (a) D($LiClO_4$)-RNase in 3.5 M $LiClO_4$ (pH 3.0), (b) D(urea)-RNase in 8 M urea (pH 3.0), and (c) N-RNase in 50 mM Gly–HCl (pH 3.0) at 4 °C. The concentration of these proteins was 10% (w/v). The spectrum of D(urea)-RNase in the 1500–1700-cm^{-1} range could not be obtained because of the large scattering by urea at 1680 and 1604 cm^{-1}.

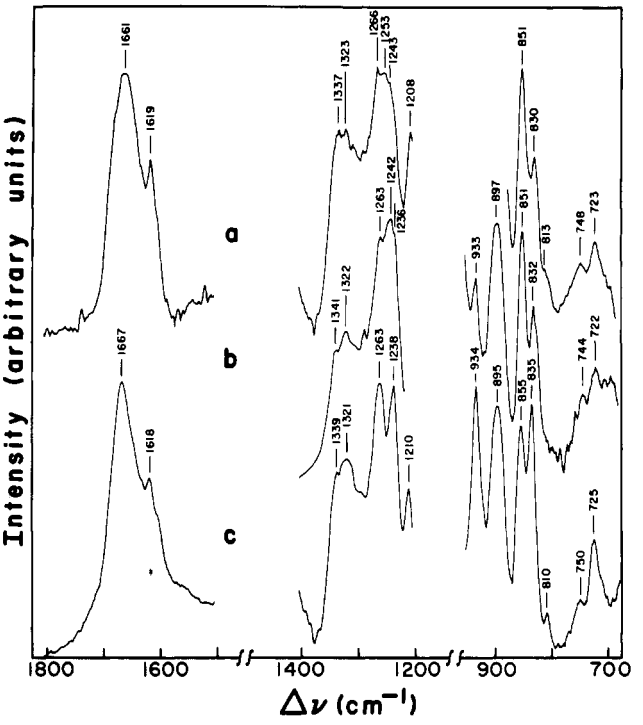

FIGURE 4: Raman spectra for (a) D($LiClO_4$)-RNase, (b) D-(urea)-RNase, and (c) N-RNase after the solvent background was subtracted from the spectra in Figure 3. Most of the Raman peaks of the solvents appeared outside of the frequency regions shown in Figure 3, and only their shoulders extended into the frequency regions shown (see especially the 900–700-cm^{-1} region of Figure 3a,b).

modes of tyrosyl side chains, and their intensity ratio is more sensitive to the state of the phenolic hydroxyl group than to the environment of the phenyl ring or to the conformation of the backbone (Siamwiza et al., 1975). Figure 4 shows that the intensity ratios of the peaks around 850 and 830 cm^{-1} are 1.55, 1.50, and 0.82 for D($LiClO_4$)-, D(urea)-, and N-RNase, respectively. The large intensity ratio for D($LiClO_4$)- and D(urea)-RNase indicates that the hydrogen bonds of three tyrosyl phenolic hydroxyl groups in N-RNase (Scheraga, 1967; Richards & Wyckoff, 1971) are disrupted in $LiClO_4$ and in urea (Siamwiza et al., 1975).

Information about the backbone structures can be obtained from the amide I and III bands around 1660 and 1250 cm^{-1}, respectively. The shift of the amide I frequency from 1667 cm^{-1} for N-RNase to 1661 cm^{-1} for D($LiClO_4$)-RNase (parts a and c of Figure 4, respectively) implies that there is some decrease in the ordered backbone structure upon addition of $LiClO_4$ (Chen & Lord, 1976). The amide I band is not observed in Figure 4b because it is masked by a strong urea peak at 1680 cm^{-1}. The peak at 1263 cm^{-1} in the spectrum of N-RNase has been assigned to the amide III vibration of the α helix, and the peak at 1238 cm^{-1} has been assigned to the corresponding vibration of the β sheet (Yu et al., 1972, 1973; Moore & Krimm, 1976; Williams & Dunker, 1981). Figure 4 shows that the ratios of the amide III *intensity* at 1263 cm^{-1} (α helix) to that at 1238 cm^{-1} (β structure) are >1 and ≈1 for D($LiClO_4$)- and N-RNase, respectively, supporting the results from the CD measurements indicating a decrease in the β-structure content of D($LiClO_4$)-RNase compared to that

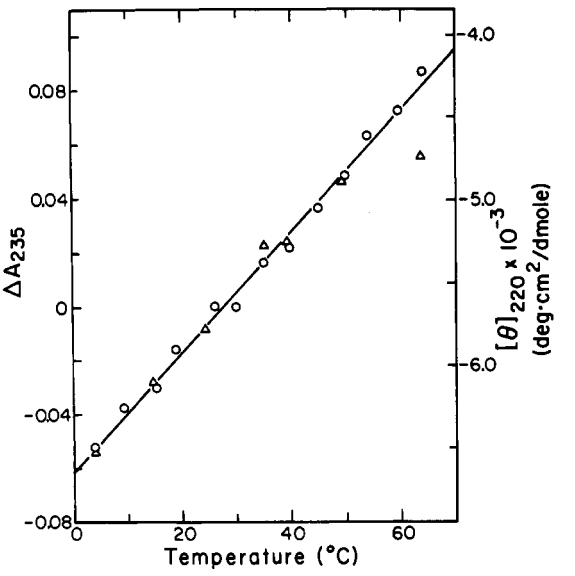

FIGURE 5: Temperature dependence of ΔA_{235} (O) and $[\theta]_{220}$ (Δ) of D($LiClO_4$)-RNase in 3.5 M $LiClO_4$ (pH 3.0). The changes in absorbance (at 235 nm) and CD (at 220 nm) were reversible in the temperature range from 4 to 64 °C. The concentration of D($LiClO_4$)-RNase is 3.4 and 3.2 × 10^{-5} M for the absorbance and CD measurements, respectively.

of N-RNase and the retention of some ordered structure that is presumably α helix. The disordered conformation of D-(urea)-RNase shows a Raman peak at 1242 cm^{-1}, which was also observed at 1250 cm^{-1} for thermally denatured RNase (Chen & Lord, 1976).

The variations of absorbance and ellipticity with temperature, for D($LiClO_4$)-RNase, are shown in Figure 5. Both vary linearly with temperature (in the range of 4–64 °C) and exhibit no evidence of a conformational transition; similar ob-

Table I: Comparison of the Conformational Behavior of D($LiClO_4$)-RNase and D(pH)-α-Lactalbumin

measurements	conformation analyzed	conformational character[a] D($LiClO_4$)-RNase	D(pH)-α-lactalbumin
CD (<240 nm)	backbone structure	~N[c]	~N
IR[b] (amide I)	backbone structure		~N
Raman (amides I and III)	backbone structure	dis~N[c]	
hydrogen-deuterium exchange	backbone structure		dis~N
CD (>250 nm)	side-chain conformation	~dis[c]	~dis
absorbance (>250 nm)	side-chain conformation	~dis	~dis
viscosity	compactness	~N	~N
luminescence	compactness		~N
X-ray scattering	compactness		~N

[a] References are Ahmad & Bigelow (1979) and this paper for D($LiClO_4$)-RNase and Kuwajima et al. (1976), Contaxis & Bigelow (1981), and Dolgikh et al. (1981) for D(pH)-α-lactalbumin. [b] Infrared spectra. [c] ~N, dis~N, and ~dis indicate that the measured conformational character is closer to the native rather than to the disordered conformation, is between the native and the disordered conformation, and is closer to the disordered rather than to the native conformation, respectively.

servations were made for D(pH)-α-lactalbumin by Dolgikh et al. (1981). Moreover, $[\theta]_{220}$ at 64 °C was 65% of the value at 4 °C; this large value suggests that the backbone conformation of D($LiClO_4$)-RNase is thermally stable in this temperature range.

Similar behavior (disordering of aromatic side chains with high retention of backbone conformation) has been reported for α-lactalbumin at low pH (Kuwajima et al., 1976; Kuwajima, 1977; Dolgikh et al., 1981). A comparison of the conformations of the denatured forms of RNase and α-lactalbumin is presented in Table I. It can be seen that the conformations of both D($LiClO_4$)-RNase and D(pH)-α-lactalbumin may be characterized as compact and thermally stable, with some ordered but flexible structure that might be α helix or helix-like but not significantly β-like and with disordered aromatic side chains.

(B) Kinetics of Unfolding of RNase and of Interconversion among Denatured Forms. With either manual mixing at 4 °C (with a dead time of 20 s) or stopped-flow mixing at 22 °C (with a dead time of 30 ms), the unfolding of N-RNase [when the solvent was changed from 50 mM Gly–HCl (pH 3.0) to 50 mM Gly–HCl plus 8 M urea (pH 3.0)] was too rapid for kinetic measurements (Nelson & Hummel, 1962). The unfolding of N-RNase was likewise too rapid for kinetic measurements when the final buffer was 50 mM Gly–HCl plus 3.5 M $LiClO_4$ (pH 3.0). However, when the concentration of $LiClO_4$ was reduced from 3.5 to 3.0 M, which is still a high enough concentration to denature RNase, then a manual-mixing experiment could be carried out to follow the change in absorption due to the unfolding of RNase at 4 °C; values of τ_{1s} = 155 s and τ_{2s} = 361 s, with f_2 = 0.47, were obtained from eq 1 and 2.

The conversions from D(urea)-RNase in 8.0 M urea to D($LiClO_4$)-RNase in 1.0 M urea and 3.5 M $LiClO_4$ and from D($LiClO_4$)-RNase in 4.0 M $LiClO_4$ to D(urea)-RNase in 7.0 M urea and 3.5 M $LiClO_4$ were too fast to be observed, even with the stopped-flow apparatus.

(C) Kinetics of Folding of RNase. With use of the manual mixing technique, both D($LiClO_4$)-RNase in 3.5 M $LiClO_4$ (pH 3.0) and D(urea)-RNase in 8 M urea (pH 3.0) were allowed to fold to N-RNase at 4 °C by changing the solvent conditions, and the kinetics were followed by the change in absorption at 287 and 235 nm and by CD measurements at 275 and 220 nm. In the folding of the $LiClO_4$- and urea-denatured proteins, two slow-folding species were observed. The same apparent rate constants and relative amplitude ratio of the two slow-folding species (for the folding of a given denatured protein) were obtained in both the absorbance and CD experiments. Similarly, two slow-folding species were observed for each protein at 22 °C in stopped-flow experiments.

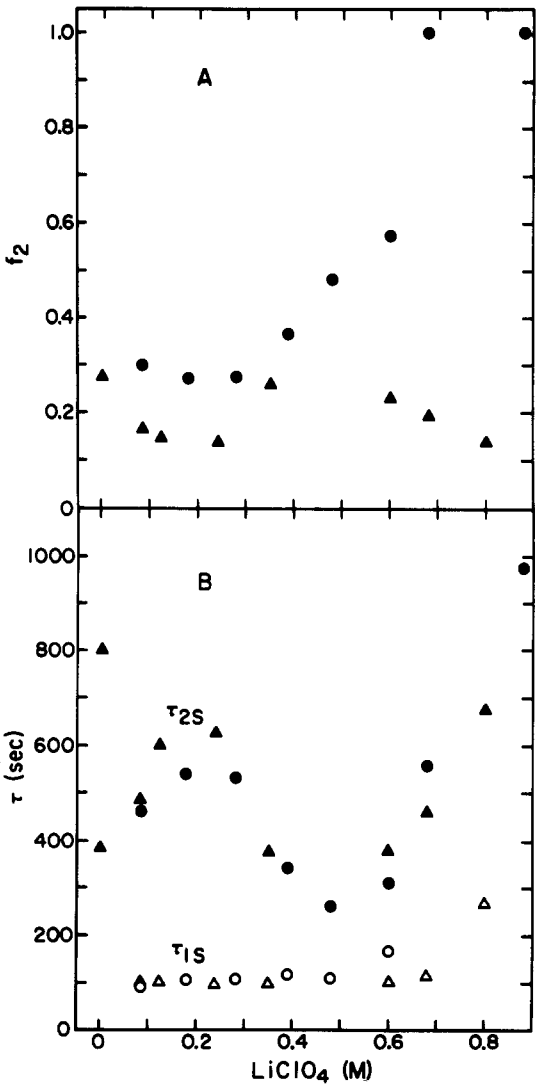

FIGURE 6: Dependence of (A) f_2 of eq 2 and (B) the time constants τ_{1s} and τ_{2s} for folding of RNase at pH 3.0 and 4 °C from 3.5 M $LiClO_4$ (○, ●) and from 8.0 M urea (△, ▲) to the final concentrations of $LiClO_4$ (0–0.9 M). The final concentration of urea was fixed at 0.50 M.

In Figure 6, f_2 and the two time constants are plotted against the *final* concentration of $LiClO_4$ in order to show how the

folding kinetics depend on the final solution conditions. The values of f_2 and the time constants for folding from D-(urea)-RNase and from D($LiClO_4$)-RNase are similar in the range of 0.085–0.6 M final concentration of $LiClO_4$. Above 0.6 M $LiClO_4$, the folding from D($LiClO_4$)-RNase exhibits only one phase (i.e., $f_1 = 0$), while the corresponding folding from D(urea)-RNase exhibits two phases.

The change in absorbance at 287 nm in the two slow-folding processes from both D($LiClO_4$)-RNase and D(urea)-RNase represented 77% of the total change in absorbance observed in the equilibrium transition. This indicates that about 77% of D($LiClO_4$)-RNase and of D(urea)-RNase folds to N-RNase at 4 °C by involvement of the two slow-folding species and 23% folds by involvement of a fast-folding species. In the CD measurements, $[\theta]_{220}$ of these two slow-folding species [in starting from D($LiClO_4$)-RNase and from D(urea)-RNase] was −4700 deg·cm²/dmol. Since $[\theta]_{220} = 0$ and −7000 deg·cm²/dmol for the disordered conformation of D(urea)-RNase and the partially ordered conformation of D($LiClO_4$)-RNase, respectively, the two kinetically trapped, slow-folding intermediates have some ordered structure, as reported by Schmid & Baldwin (1979) for a similar process starting from D-(Gdn·HCl)-RNase, but are less ordered than D($LiClO_4$)-RNase.

Discussion

(A) Nature of a Denatured Protein. It is useful to begin the Discussion by defining the conformational states of a protein. When a native protein is subjected to denaturing conditions such as high temperature, extremes of pH, urea, neutral salts, organic solvents, etc., the protein can exhibit an overall transition. The conformational states before and after the transition are defined as *native* and *denatured* states, respectively. Minor conformational changes that occur outside of the transition region and are accompanied by small changes in ΔH and ΔS compared to the large ones that are characteristic of overall denaturation are defined as *fluctuations* or *isomerizations* of the native or denatured conformations. Such fluctuations or isomerizations have been observed by detection procedures that are sensitive to small local conformational changes (Hirs, 1962; Rupley & Scheraga, 1963; Roberts et al., 1969; Zaborsky & Milliman, 1972; Burgess & Scheraga, 1975; Chen & Lord, 1976; Matheson & Scheraga, 1979a,b). When a transition curve is plotted, the properties (such as absorbance, CD, viscosity, etc.) before and after the transition, i.e., the properties of the native and denatured conformations, can be reasonably extrapolated into the folded, unfolded, and transition regions. Species whose properties are distinct from those of the native and denatured species or from their extrapolated values are defined as *intermediates*; e.g., Schmid & Baldwin (1979) detected a hydrogen-bonded intermediate in the kinetic folding from D(Gdn·HCl)-RNase to N-RNase.

These simple definitions are applicable to *small* globular proteins in general. If a protein contains more than one domain, and the cooperativity between the domains is low, it may be possible to study the native/denatured states of each domain instead of the whole protein. Here, we emphasize the fact that denatured conformations do not mean disordered conformations, as described in the introduction. Actually cooperative conformational transitions between different types of denatured conformations are often observed; e.g., D($LiClO_4$)-RNase shows a cooperative change in $[\theta]_{220}$ upon addition of urea (Ahmad & Bigelow, 1979). These transitions between denatured conformations are sometimes easily misinterpreted as those between *intermediates* and denatured conformations (Kuwajima et al., 1976). Figure 7A is a schematic phase diagram indicating the regions of stability of D($LiClO_4$)-, D(urea)-, and N-RNase, on the basis of the data of Ahmad & Bigelow (1979). Figure 7B is a schematic hypothetical curve showing the variation of $[\theta]_{220}$ as urea is added to a solution of N-RNase at a fixed concentration of $LiClO_4$ (corresponding to the horizontal dashed line of Figure 7A). In 1.8 M $LiClO_4$ (pH 3.0) at 25 °C (point 1 in Figure 7A), N-RNase adopts a predominantly native conformation because 1.8 M $LiClO_4$ by itself is not a high enough concentration to denature RNase. Addition of a small amount of urea (1.5 M) (point 2 in Figure 7A) *destabilizes* the native conformation, producing D($LiClO_4$)-RNase. Further, D($LiClO_4$)-RNase shows a transition to D(urea)-RNase when the concentration of urea is increased further (point 3 in Figure 7A). Since these transitions are induced only by increasing the urea concentration, the observed two transitions might be misinterpreted as N-RNase → I(urea)-RNase → D(urea)-RNase instead of N-RNase → D($LiClO_4$)-RNase → D(urea)-RNase, i.e., by misinterpreting D($LiClO_4$)-RNase as I(urea)-RNase. This misinterpretation is avoided by knowing the phase diagram. These complications arise from the fact that various denaturing agents enhance each other's effect in unfolding a protein. Since many factors such as temperature, pH, pressure, ionic strength, etc. can act as protein denaturants, one must identify which of these factors is effective in denaturing the protein. The phase diagram, which represents the conformational states of the protein under different denaturing conditions, is useful for this identification. However, a complete phase diagram has n dimensions (where n is the number of denaturing factors) and is very complicated; hence the phase diagram is usually expressed in two dimensions ($n = 2$) by fixing the other ($n - 2$) factors close to those of physiological conditions (Kuwajima et al., 1976; Ahmad & Bigelow, 1979). With the aid of these phase diagrams, the conformational states of a protein can be defined under different conditions.

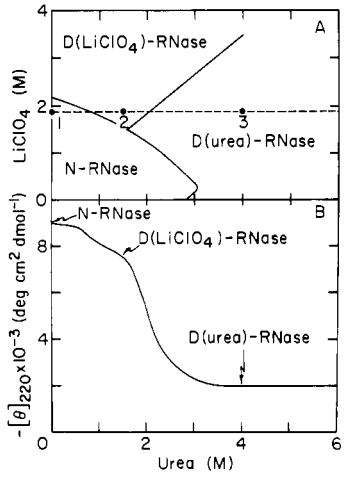

FIGURE 7: (A) Schematic phase diagram at 25 °C showing the regions of stability of D($LiClO_4$)-, D(urea)-, and N-RNase as a function of the concentrations of $LiClO_4$ and urea, on the basis of the data of Ahmad & Bigelow (1979). The solid lines are the transition regions between the species. The dashed line of constant [$LiClO_4$] is the basis for drawing the curve in part B. Points 1, 2, and 3 represent illustrative urea concentrations at which D($LiClO_4$)-, D(urea)-, and N-RNase are stable, at the given value of [$LiClO_4$]. (B) A hypothetical schematic curve for the change of $[\theta]_{220}$ implied by a change in [urea] along the dashed line in part A. The dominant conformations at various values of [urea] are indicated.

(B) Effects of Initial and Final Solution Conditions on the Folding Pathways. Figure 8 shows schematic kinetic folding pathways (under folding conditions) starting from two different denatured conformations; i.e., unfolding from the native

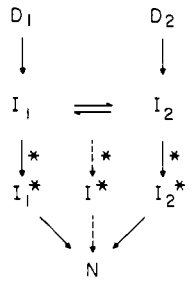

FIGURE 8: Schematic representation of the folding pathways. D_1 and D_2 are two different denatured conformations. I_1 and I_2 are the "Intermediates" produced from D_1 and D_2, respectively, by a change of solvent from a denaturing one to a folding one. The rate-limiting steps of the pathways are expressed by arrows with asterisks. If the interconversion between I_1 and I_2 is much faster than the formation of the native protein (N), a preequilibrium state is attained between I_1 and I_2, and N is produced from the "Intermediates" in the preequilibrium through "Intermediate*" I* (expressed by dashed arrows). If the interconversion between I_1 and I_2 is much slower than the formation of N, "Intermediates*" I_1* and I_2* are produced from I_1 and I_2, respectively, and are converted to N (expressed by solid arrows).

conformation is ignored. Two denatured conformations, D_1 and D_2, are converted to "Intermediates" I_1 and I_2, respectively, by a change in solvent. If the interconversion between I_1 and I_2 is much faster than the formation of the native protein (N), a preequilibrium state, which is controlled by the final solution conditions, is attained between I_1 and I_2 (Konishi et al., 1981), and "Intermediate*" I* and N are produced from the "Intermediates" in preequilibrium through the pathway represented by the dashed lines. Therefore, the pathway is independent of the initial denatured conformation and is controlled by the final solution conditions. Examples of such a mechanism would be the similar folding starting from D-($LiClO_4$)-RNase and D(urea)-RNase to N-RNase in 0.5 M urea and less than 0.6 M $LiClO_4$ (pH 3.0) (Figure 6) and the similar folding of lysozyme starting from D(acetic acid)- and D(Gdn·HCl)-lysozyme (Kato et al., 1981).

If the interconversion between I_1 and I_2, however, is much slower than the formation of N, the preequilibrium state between I_1 and I_2 is not attained, so that "Intermediates*" I_1* and I_2* are produced from I_1 and I_2, respectively, with different rate constants, and then N is produced from I_1* and I_2* through the pathways represented by the solid lines. Therefore, the pathways from I_1 and I_2 depend on the initial denatured conformations, D_1 and D_2. The observed three phases, one fast and two slow ones, in the kinetic folding from D(Gdn·HCl, urea, or temperature)-RNase (Baldwin & Creighton, 1980) and the two slow phases in Figure 6 would be examples of this situation because these various phases arise from differences in the initial denatured conformations (Konishi et al., 1982b).

(C) Role of Ordered Structure in D($LiClO_4$)-RNase in the Folding Pathway. It was anticipated that the ordered structure in D($LiClO_4$)-RNase might play a role in the folding pathway, because Schmid & Baldwin (1979) reported that the kinetically trapped *intermediate* in the slow-folding process from D(Gdn·HCl)-RNase has hydrogen bonds and disordered tyrosyl side chains, which are similar in character to those of D($LiClO_4$)-RNase. As in the case of RNase, a kinetically trapped bovine carbonic anhydrase intermediate (under folding conditions) shows a nativelike CD spectrum in the far-UV region and a disordered-like CD spectrum in the near-UV region (McCoy et al., 1980). However, the slow-folding intermediate species in the conversion from D(urea)-RNase to N-RNase is *not* the same as partially ordered D($LiClO_4$)-RNase. If it were, then D($LiClO_4$)-RNase should exhibit only a slow-folding process. Since both a fast-folding process (23%) and a slow-folding process (77%) were observed in the conversion from both D($LiClO_4$)-RNase and D(urea)-RNase to N-RNase, it appears that the partially ordered conformation of D($LiClO_4$)-RNase does not comprise the nucleation sites that are formed in the rate-limiting steps (see the introduction) and has no effect in enhancing the kinetic folding of RNase. Moreover, the large negative value of $[\theta]_{220}$ (=−7000 deg·cm^2/dmol) of D($LiClO_4$)-RNase compared to $[\theta]_{220}$ (=−4700 deg·cm^2/dmol) of the two slow-folding intermediates indicates that some ordered structure in D($LiClO_4$)-RNase is disordered in the early stages of the slow-folding pathways; i.e., when D($LiClO_4$)-RNase is first converted to I($LiClO_4$)-RNase in the slow-folding process, there is a decrease in order and then an increase in going to N-RNase.

Although the ordered structure in D($LiClO_4$)-RNase has not been identified, the data in Table I indicate a high retention of α-helix content and a low β-structure content in D($LiClO_4$)-RNase. Because of the compact structure of D($LiClO_4$)-RNase, some β-bend structure might be involved in the protein. These α helix and β bends are formed by short- and medium-range interactions. Thus, short- and medium-range interactions might not be sufficient to form the nucleation sites, and some long-range interactions such as extended structures might play a significant role in formation of the nucleation sites.

Conclusions. D($LiClO_4$)-RNase appears to have a high α-helix content and a low β-structure content and a compact overall structure. The kinetic folding from D($LiClO_4$)-RNase to N-RNase was compared to that from D(urea)-RNase. The similar kinetic folding behavior from the different denaturing conditions demonstrated that the ordered structure in D($LiClO_4$)-RNase is not sufficient to form the nucleation sites.

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