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

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ABSTRACT: Bovine pancreative ribonuclease A (RNase A), denatured by 3.5 M $LiClO_4$ (pH 3.0), has some ordered conformation as indicated by a high retention of α-helix and compact structure. This effect of $LiClO_4$ was confirmed by the observation that the α-helix of isolated S-peptide is stabilized in the presence of 3.5 M $LiClO_4$ (pH 3.0), as measured by circular dichroism and nuclear magnetic resonance. The effect of the retained α-helices and compact structure on the folding kinetics of RNase A was studied by comparison with the kinetic folding from urea-denatured RNase A, which has no ordered structure. In contrast to our previous study under *folding conditions* [Denton, J. B., Konishi, Y., & Scheraga, H. A. (1982) *Biochemistry 21*, 5155], the kinetic folding/unfolding experiments were carried out here in the *transition regions* between native and $LiClO_4$-denatured RNase A and between native and urea-denatured RNase A. The measured relaxation times were extrapolated to the triple point, where native RNase A, $LiClO_4$-denatured RNase A, and urea-denatured RNase A have the same thermodynamic stability and are at the same concentration, in order to compare the rates of these two processes under the same solvent conditions. Under these conditions, both folding and unfolding pathways are studied simultaneously without any accumulated intermediates. No significant acceleration of folding was observed from $LiClO_4$-denatured RNase A as compared to that from urea-denatured RNase A. This indicates that all ordered structures in RNase A are not equivalent in their influence on the folding pathway; some may play an essential role and some may not. It appears that the α-helix in the S-peptide portion of RNase A and one or more β-bends, which establish the compact structure, are among those ordered structures that do not play an essential role in the folding of RNase A.

The stability, and hence biological function, of a protein depends on the interatomic interactions that lead to the native conformation. These conformations, and the interactions that lead to them, are studied primarily by X-ray diffraction of protein crystals and, for example, by nuclear magnetic resonance (NMR)[1] spectroscopy of protein solutions. These interactions, and the specific conformations that they generate, determine how the newly synthesized polypeptide chain (in vivo) folds to the native structure. Konishi et al. (1982c) proposed that nonnative, as well as native, interactions play significant roles in these folding pathways depending on the nature of each protein and the solution conditions. A typical example of the importance of a nonnative interaction for folding was reported for bovine pancreatic trypsin inhibitor (Creighton, 1978); i.e., a nonnative disulfide bond between Cys-5–Cys-14 or Cys-5–Cys-38 was formed in order to regenerate the protein from the reduced form under the conditions used in Creighton's experiments.

Since it is difficult to characterize disordered conformations, we focus our attention here only on ordered ones (α-helices, extended structures, and β-turns) and the role that they play in determining the folding pathway. Short- and medium-range interactions are sufficient to stabilize such ordered structures (Kotelchuck & Scheraga, 1968; Finkelstein & Ptitsyn, 1971; Scheraga, 1973; Anfinsen & Scheraga, 1975), but long-range interactions as well as short- and medium-range interactions are required to stabilize the β-sheets formed from extended structures. Tanaka & Scheraga (1975, 1977) proposed that structures that form in response to short- and medium-range interactions would be established predominantly in the early stages of the folding pathway. This model was generalized by Wako & Saitô (1978) and Gō & Abe (1981). When this model was applied to RNase A,[1] it was shown that α-helices and β-turns should form during the early stages of the folding pathway (Némethy & Scheraga, 1979; Miyazawa & Jernigan, 1982). A kinetic experiment also supported this model by demonstrating that the α-helix in the N-terminal region is folded prior to the folding of other parts of the polypeptide (Blum et al., 1978). There arises, however, the question as to whether such ordered structures that form early in the folding process actually accelerate folding; this is a question that can be answered by kinetic experiments.

The S-peptide of RNase contains an α-helix in its native conformation, and its effect on the folding pathway has been studied by using S-protein and S-peptide (Labhardt & Baldwin, 1979a,b; Labhardt et al., 1983). It was demonstrated that S-peptide forms a reversible complex with S-protein at an early stage of folding and that the kinetics of refolding of S-protein are accelerated by the presence of S-peptide (Labhardt & Baldwin, 1979a,b; Labhardt et al., 1983). This indicates that S-peptide plays a significant role in the folding pathway of *RNase S*. However, it is desirable to use *RNase A* to analyze the roles that the ordered structures (formed at an early stage of folding) play in the folding pathways of RNase A, because cleavage of the peptide bond between S-peptide and S-protein may affect the folding pathway (Acharya & Taniuchi, 1982). Specifically, we shall examine whether or not the α-helix of the S-peptide portion of RNase A or β-turns in other parts of the structure accelerate folding.

Konishi et al. (1981, 1982a) and Denton et al. (1982) showed the general applicability of a preequilibrium treatment

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; N-RNase A, native RNase A; D(urea)-RNase A, RNase A denatured by urea (the denaturant is enclosed in parentheses); S-peptide, N-terminal peptide (residue 1–20) of RNase A; Gdn·HCl, guanidine hydrochloride; CD, circular dichroism; NMR, nuclear magnetic resonance; UV, ultraviolet; Gly, glycine; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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of the kinetics of protein folding. The preequilibrium treatment leads to the following kinetic equation for the rate of formation of native protein along the i pathways:

$$dN/dt = \sum_i k_i f_i (1 - N) \quad (1)$$

where f_i is the fraction of a prenucleated "Intermediate" i as compared to the total "Intermediates". N is the fraction of refolded protein; i.e., $(1 - N)$ is the fraction of the total "Intermediates", where we ignore the accumulation of "Intermediates*"[2] after the rate-limiting steps[3] because they are converted rapidly to N after their slow formation (except under some special conditions). Thus, there are three ways for ordered structures to contribute to the kinetics of folding. (a) The first is by forming prenucleation site(s). This increases f_i in eq 1 so that the formation of N is accelerated. (b) The second is by forming nucleation site(s) [*during* the rate-limiting step(s)]. This decreases the activation free energy, thereby increasing the value of k_i for the rate-limiting step(s); thus, dN/dt is increased. (c) The third is by forming "Intermediates*"; i.e., the species classified as an "Intermediate*" is converted rapidly to N without passing through the rate-limiting step.

In this paper, we shall analyze the effects of the α-helices and compact structure on the conformations or stabilities of the prenucleation and the nucleation site(s); i.e., we shall consider effects a and b together and determine whether ordered structures accelerate folding (effect c is excluded by the argument presented below). To assess these effects, we prepared a partially ordered RNase A in which the fraction of α-helix and the compactness are close to those of native RNase A (Ahmad & Bigelow, 1979; Denton et al., 1982) and then studied the rate of folding from such a partially ordered RNase A in comparison to that from completely disordered RNase A, viz., urea-denatured RNase A.

In kinetic refolding experiments, one usually uses folding conditions as the final solution conditions; then only the native conformation can exist as a stable form. Denton et al. (1982) also used folding conditions as their final ones and found that both D($LiClO_4$)-RNase A and D(urea)-RNase A[4] are converted very rapidly to kinetically trapped "Intermediates" by the solvent jump and then the native conformation is formed from such semistable conformations. The rapid conversion of D($LiClO_4$)-RNase A to "Intermediates" clearly excluded the possibility that D($LiClO_4$)-RNase A exists as "Intermediates*"; hence, effect c above can be excluded from consideration. Circular dichroism (CD) measurements indicated the presence of some ordered backbone structures in the kinetically trapped "Intermediates", but the degree of order was less than that of D($LiClO_4$)-RNase A (Denton et al., 1982). Thus, the refolding pathway is composed of at least three sequential species, viz., a denatured conformation, kinetically trapped "Intermediates", and the native conformation.

It is not easy, however, to determine the conformations of the kinetically trapped "Intermediates" simply because such semistable species cannot be isolated. Therefore, in contrast to our previous studies under folding conditions (Denton et al., 1982), we carry out the folding experiments here under conditions where such "Intermediates" do not exist. If the final solution conditions in the kinetic refolding experiments are those corresponding to a transition point between denaturing and native conditions, then the process can be treated as one in which only native and denatured species exist and in which intermediates (including kinetically trapped ones) do not accumulate, because the equilibrium transition between native RNase A and D(urea)- or D($LiClO_4$)-RNase A is highly cooperative[4] (Creighton, 1979; Ahmad & Bigelow, 1979), and also because there is a large activation energy for the interconversion of native and denatured species. The transition between D(urea)-RNase and D($LiClO_4$)-RNase A can also be treated as a two-state one because of the cooperativity of the transition (Ahmad & Bigelow, 1979). In addition, since the folding and unfolding pathways are reversible in the transition region, we may obtain information about both the folding and unfolding pathways under the same solvent conditions. Furthermore, from studies at the triple point, where native RNase A, D(urea)-RNase A, and D($LiClO_4$)-RNase A have the same stability and are at the same concentration, we may learn about the activation free energies for interconversion between these three species under the same conditions; this would reveal the effects of the α-helices and the compact structure of D($LiClO_4$)-RNase A on the kinetics of folding of RNase A.

Experimental Procedures

Materials. The same materials were used as in the paper by Denton et al. (1982) except for the following. Bovine pancreatic ribonuclease S-peptide was purchased from Sigma Chemical Co. and used without further purification. *n*-C_4H_9OD (98 atom % D) was obtained from Aldrich Chemical Co. MES was obtained from Sigma Chemical Co. Bio-Gel P-6 resin was purchased from Bio-Rad Laboratories. All solutions, except those used for NMR measurements, were buffered with 50 mM Gly·HCl at pH 3.0 and were clarified routinely with 0.45-μm filters purchased from Millipore Corp. The solutions for NMR measurements were prepared in D_2O (95.8 atom % D; Aldrich Chemical Co.) and adjusted to pD 3.0 with 5.5 N DCl or 10 N NaOD.

Concentrations. Protein concentrations were determined spectrophotometrically by using $\epsilon_{275} = 9300\ M^{-1}\ cm^{-1}$ or $\epsilon_{277.5} = 9800\ M^{-1}\ cm^{-1}$ for native RNase A (Sela & Anfinsen, 1957) and $\epsilon_{275} = 9000\ M^{-1}\ cm^{-1}$ for D(urea)- and D($LiClO_4$)-RNase A (Denton et al., 1982). The concentration of S-peptide in 50 mM Gly·HCl buffer (pH 3.0) was also determined spectrophotometrically by using $\epsilon_{257} = 290\ M^{-1}\ cm^{-1}$ (Klee, 1968). The extinction coefficient of S-peptide at a high concentration of $LiClO_4$ was estimated as follows: Two identical aliquots of a concentrated S-peptide solution were diluted to the same final volume to obtain solutions of S-peptide in 3.3 M $LiClO_4$ and in 50 mM Gly·HCl buffer, respectively, at the same peptide concentration. By use of $\epsilon_{257} = 290\ M^{-1}\ cm^{-1}$ for S-peptide in 50 mM Gly·HCl buffer, a value of $290\ M^{-1}\ cm^{-1}$

[2] "Intermediates" and "Intermediates*" represent the intermediates prior to and after, respectively, the rate-limiting step[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 pathways. Any "Intermediate" that takes part in a rate-limiting step is defined as a prenucleated "Intermediate" in order to distinguish it from other "Intermediates". The term "nucleation" is reserved for those sites formed *during* the rate-limiting step.

[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, extreme of pH, urea, Gdn·HCl, neutral salt, organic solvents, etc. The nonnative conformation is not necessarily disordered. The details are discussed by Denton et al. (1982).

was found for ϵ_{257} in 3.3 M $LiClO_4$ as well. Since ϵ_{257} of S-peptide is independent of the concentration of $LiClO_4$ in this range, we used the same value in 3.3 and 3.5 M $LiClO_4$.

Spectroscopic Measurements. Absorption spectra were measured on an HP 8450 UV/vis spectrophotometer or on a modified Cary Model 14 spectrophotometer (Denton et al., 1982). CD measurements were made on a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment. ^1H NMR spectra were obtained on a Bruker 300 MHz Model 300 instrument, in which a 5-mm NMR tube was used and the temperature of the sample was adjusted[5] to 25 ± 2 °C. The concentrations of RNase A and S-peptide were 1.5 and 1.8 mM, respectively. The spectra are the results of 500–1000 4.0-s scans, and an internal proton lock to the residual HDO peak was used to stabilize the field.

Equilibrium Measurements. Since RNase A is denatured by high concentrations of urea and of $LiClO_4$ [providing D(urea)-RNase A and D($LiClO_4$)-RNase A, respectively], Ahmad & Bigelow (1979) obtained a phase diagram composed of N-, D(urea)-, and D($LiClO_4$)-RNase A phases as a function of the concentrations of urea and $LiClO_4$. Since our kinetic experiments were performed at transition points, where the rates are sensitive to solvent composition, we had to redetermine the phase diagram precisely. An aliquot (90 μL) of concentrated RNase A solution (1.24 mM) in 50 mM Gly·HCl buffer (pH 3.0) was added to 2.91 mL of a solvent composed of various concentrations of urea and of $LiClO_4$ in 50 mM Gly·HCl (pH 3.0). The final protein concentration was 37.3 μM. After the protein reached conformational equilibrium (by incubation for more than several hours), the absorbance at 235 and 275 nm and the CD at 220 nm of the solution were measured.

Kinetic Measurements. The kinetics of folding/unfolding of RNase A were measured as follows. An aliquot (90 μL) of concentrated RNase A solution (1.24 mM) in 50 mM Gly·HCl buffer (pH 3.0), in 8 M urea (pH 3.0), or in 3.5 M $LiClO_4$ (pH 3.0) was added to 2.91 mL of a solvent containing various concentrations of urea and of $LiClO_4$, which was kept stirred by an electrically driven stirring rod. The final protein concentration was 37.3 μM, and the concentrations of urea and $LiClO_4$ corresponded to transition regions. After mixing for 15 s, the kinetics were followed by recording the change of absorbance at 235 nm for about 10 times longer than the relaxation time of the process. The data were digitized every second; i.e., the data were collected for 1 s and averaged, with an analog-to-digital converter connected to the spectrophotometer and then input directly to a Prime 350 computer for a kinetic analysis as described under Results.

Selective Deuteration. Selective deuteration of the His C-2 protons of RNase A with 0.7 M n-C_4H_9OD in D_2O was carried out in order to assign the His C-2 proton resonances by using a modification of the procedure of Bierzynski & Baldwin (1982). MES buffer was substituted for sodium cacodylate, and the product was desalted on Bio-Gel P-6 with 0.1 N acetic acid and lyophilized before use.

Results

Effect of $LiClO_4$. Figure 1 shows the CD spectra of S-peptide in 50 mM Gly·HCl buffer (pH 3.0) and in 3.5 M $LiClO_4$ (pH 3.0) at 22 °C. Since the spectra in Figure 1 are typical ones for mixtures of α-helix and random coil (Greenfield & Fasman, 1969) and S-peptide is known to form an α-helix[5] (Tamburro et al., 1968; Klee, 1968; Silverman et al., 1972), with S-protein (Wyckoff et al., 1970) and in RNase A (Wlodawer & Sjolin, 1983), the CD spectrum (207–240 nm) was decomposed into fractions of α-helix and random coil by using the methods of Chen et al. (1974) and Labhardt (1982). The length of helix was taken as 11 residues,[6] as observed in the crystal structure of RNase A (Borkakoti et al., 1982; Wlodawer & Sjolin, 1983; Borkakoti, 1983). The results in Table I show that 3.5 M $LiClO_4$ (pH 3.0) is a moderate helix supporting solvent although not as strong as trifluoroethanol (Tamburro et al., 1968).

Blum et al. (1978) showed that the resonance of the His-12 C-2 proton in S-peptide shifts upfield when S-peptide forms an α-helix. The addition of $LiClO_4$ to S-peptide solution in D_2O (pD 3.0) induced an upfield shift of the His-12 C-2 proton resonance (Figure 2A,B).[5] This supports the conjecture (Ahmad & Bigelow, 1979; Denton et al., 1982) that high concentrations of $LiClO_4$ stabilize α-helices.

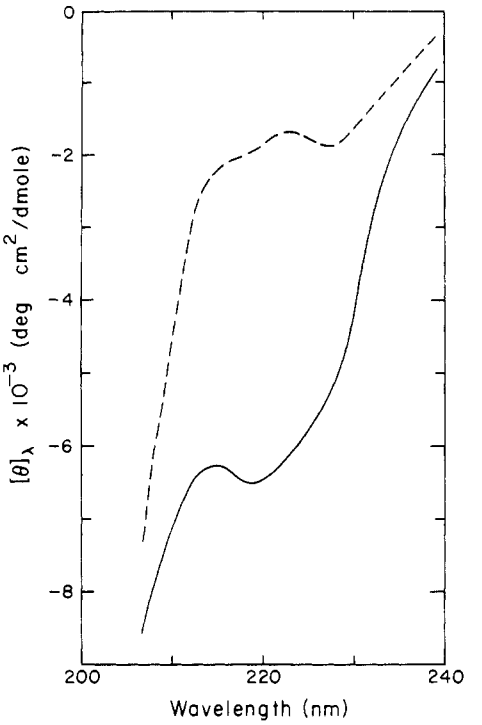

FIGURE 1: Circular dichroism spectra of S-peptide (0.15 mM) in 50 mM Gly·HCl (pH 3.0) (---) and in 3.5 M $LiClO_4$ and 50 mM Gly·HCl (pH 3.0) (—) at 22 °C.

Table I: Helix Content of S-Peptide in Various Solvents at 22 °C

solvent	% helix (20 residues)[a]	% helix (11 residues)[a]
50 mM Gly·HCl buffer (pH 3.0)	0	0
3.5 M $LiClO_4$ (pH 3.0)	11	24
trifluoroethanol	35[b]	73[b]

[a] In N-RNase A, only 11 of the 20 residues of S-peptide are in a helical state. Thus, the values in the third column are a better reflection of the helix content of S-peptide in a given solvent. [b] The data of Tamburro et al. (1968) were analyzed by using the methods of Chen et al. (1974) and Labhardt (1982).

[5] The S-peptide is preferentially helical in water at low temperature but not at room temperature (Klee, 1968; Silverman et al., 1972). The purpose of carrying out the CD and NMR experiments at room temperature was to determine whether $LiClO_4$ induces the formation of helix at this temperature.

[6] The basis spectra of Chen et al. (1974) are dependent on helix length, and account of this length dependence was taken in analyzing the CD spectra.

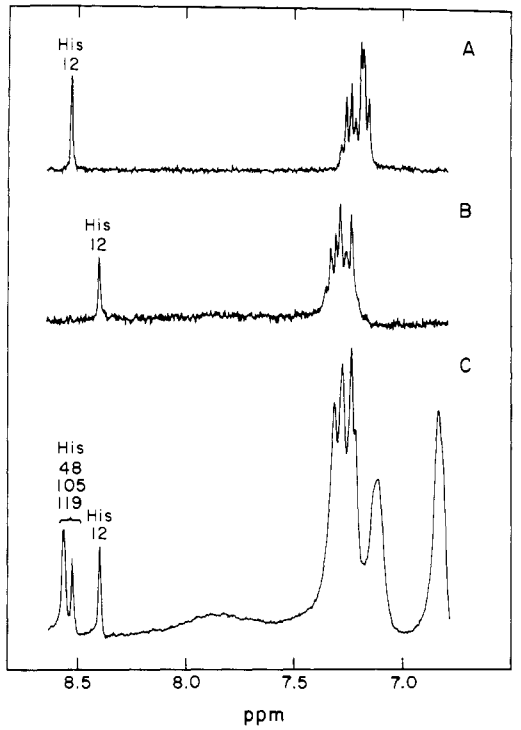

FIGURE 2: ^1H NMR spectra for S-peptide (1.86 mM) (A) in D_2O (pD 3.0) and (B) in 3.5 M $LiClO_4/D_2O$ (pD 3.0) at 25 °C. (C) ^1H NMR spectrum for D($LiClO_4$)-RNase A (1.83 mM) in 3.5 M $LiClO_4/D_2O$ (pD 3.0) at 25 °C. Chemical shifts are relative to the internal standard DSS.

Ahmad & Bigelow (1979) and Denton et al. (1982) indicated that D($LiClO_4$)-RNase A has a compact structure with a high retention of α-helix. Here we measured the NMR spectrum of D($LiClO_4$)-RNase A to obtain more information about the existence of the α-helix (Figure 2C). The resonances of the His C-2 protons are split into two distinct sets of peaks (8.35 and 8.55 ppm from DSS). Since the relative areas of these two resonances are 1:3, one histidine is involved in a specific environment substantially different from the other three histidine residues. This histidine residue, with a high field chemical shift, was assigned to His-12 by selective deuteration of the His C-2 protons (Bierzynski & Baldwin, 1982) as follows. Figure 3B shows the NMR spectrum of partially deuterated RNase A under native conditions, where each histidine C-2 proton peak has a different area relative to the unexchanged His-105 C-4 proton because the C-2 protons exchange at different rates and this spectrum was taken on material obtained at a time when the various protons had exchanged to different extents (Bierzynski & Baldwin, 1982). Since the histidine C-2 protons in Figure 3 were deuterated to the same extent, then the correspondence between the areas of the peaks labeled "His 12" in Figure 3 identifies this peak as the one corresponding to the His-12 C-2 proton. Furthermore, since the chemical shift of the His-12 C-2 proton in D($LiClO_4$)-RNase A is similar to that of S-peptide in 3.5 M $LiClO_4$, we assign His-12 as part of an α-helix, which is probably formed at residues 3–13. Thus, D($LiClO_4$)-RNase A has α-helices (especially in the N-terminal region) and a compact structure (Ahmad & Bigelow, 1979; Denton et al., 1982).

Phase Diagram. In order to obtain the phase diagram relating the various species in the system, the fractions of N-, D(urea)-, and D($LiClO_4$)-RNase A, f_N, $f_{D(urea)}$, and $f_{D(LiClO_4)}$, respectively, were estimated as follows. Since the values of ϵ_{275} for N-RNase A (9300 M^{-1} cm^{-1}) and for D(urea)- and D($LiClO_4$)-RNase A (9000 M^{-1} cm^{-1}) are so close, we used

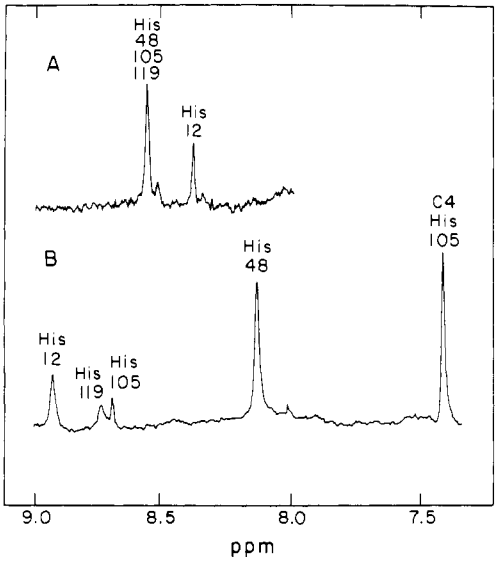

FIGURE 3: ^1H NMR spectra of (A) D($LiClO_4$)-RNase A in 3.5 M $LiClO_4/D_2O$ (pD 3.0) and (B) N-RNase A in D_2O (pD 3.0). The His C-2 protons of RNase A were partially deuterated as described in the text, and the sample was lyophilized; portions of this same partially deuterated protein were dissolved in $LiClO_4$ and in D_2O, respectively. The solution of N-RNase A in D_2O was heated at 60 °C for 10 min to exchange the amide protons with deuterium. Then the spectra in (A) and (B) were obtained. The C2 resonances of N-RNase A (B) are those of His-12, -119, -105, and -48, and the C4 is that of His-105, in the direction from downfield to upfield (Bierzynski & Baldwin, 1982). The resonances of D($LiClO_4$)-RNase A are assigned from the peak areas of partially deuterated His residues. The concentrations of N-RNase A and D($LiClO_4$)-RNase A were both 2.92 mM. Chemical shifts are relative to the internal standard DSS.

ϵ_{275} = 9100 M^{-1} cm^{-1} as the average of the three values to determine protein concentrations in the three regions. (There is an isosbestic point near 275 nm, making this a suitable wavelength to determine protein concentration.) Since ϵ_{235} and $[\theta]_{220}$ are sensitive to conformational changes of RNase A (Ahmad & Bigelow, 1979; Denton et al., 1982), the conformational transitions were followed by changes of ϵ_{235} and $[\theta]_{220}$. Assuming that urea and $LiClO_4$ affect ϵ_{235} and $[\theta]_{220}$ of the three conformations independently, the solvent dependences of ϵ_{235} and $[\theta]_{220}$ were measured for N-, D(urea)-, and D($LiClO_4$)-RNase A separately, i.e., under conditions where only one of these species exists and the concentrations of the other two are negligibly small. For N-RNase A (under folding conditions where N is stable), the dependence of ϵ_{235} (M^{-1} cm^{-1}) on the concentrations of urea and $LiClO_4$ is

$$\epsilon_{235} = (3.87 \times 10^4) + (1.23 \times 10^2)[\text{urea}] - (1.44 \times 10^3)[LiClO_4] \quad (2)$$

where 3.87×10^4 is the value in the absence of urea and $LiClO_4$, 1.23×10^2 is the slope of the curve for the dependence of ϵ_{235} on urea concentration *before* the urea-induced transition, and -1.44×10^3 is the corresponding slope *before* the $LiClO_4$-induced transition. However, $[\theta]_{220}$ was found to be independent of the urea and $LiClO_4$ concentrations in the pretransition region, i.e.

$$[\theta]_{220} = -8.00 \times 10^3 \quad (3)$$

In a similar way, ϵ_{235} for D(urea)-RNase A (under urea-denaturing conditions in the presence and absence of $LiClO_4$) was found to be

$$\epsilon_{235} = (2.90 \times 10^4) + (3.23 \times 10^2)[\text{urea}] - (1.96 \times 10^2)[LiClO_4] \quad (4)$$

where 2.90×10^4 is obtained by extrapolating the values *after*

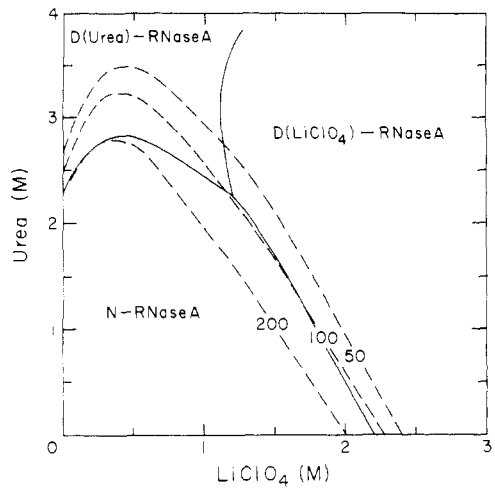

FIGURE 4: Phase diagram for N-, D($LiClO_4$)-, and D(urea)-RNase A as a function of urea and $LiClO_4$ concentrations at 22 °C. The solid lines are the loci of the midpoints of the transition curves along which two of the three species are in equilibrium. The curves intersect at the triple point ([urea] = 2.25 M and [$LiClO_4$] = 1.20 M), where N-RNase A, D($LiClO_4$)-RNase A and D(urea)-RNase A are all at equal concentrations in equilibrium. The dashed lines represent constant values of the relaxation time of the major unfolding/folding kinetic phase. Some of the values of the relaxation times (in seconds) are given on the dashed lines. This diagram is based on 136 kinetic data points under various solvent conditions.

the urea-induced transition to [urea] = [$LiClO_4$] = 0 and 3.23×10^2 and -1.96×10^2 are the slopes of the curves obtained by adding urea and $LiClO_4$, respectively, to D(urea)-RNase A. Again, $[\theta]_{220}$ was found to be independent of the urea and $LiClO_4$ concentrations in the region *after* the urea-induced transition, i.e.

$$[\theta]_{220} = -1.70 \times 10^3 \quad (5)$$

Finally, ϵ_{235} for D($LiClO_4$)-RNase A (under $LiClO_4$-denaturing conditions in the presence and absence of urea) was found to be

$$\epsilon_{235} = (2.96 \times 10^4) + 6[\text{urea}] - (3.92 \times 10^2)[LiClO_4] \quad (6)$$

where 2.96×10^4 was obtained by extrapolating the values *after* the $LiClO_4$-induced transition to [$LiClO_4$] = [urea] = 0, and 6 and -3.92×10^2 are the slopes of the curves obtained by adding urea and $LiClO_4$, respectively, to D($LiClO_4$)-RNase A. For ($LiClO_4$)-RNase A, however, $[\theta]_{220}$ depends on the urea concentration

$$[\theta]_{220} = (-7.00 \times 10^3) + (1.22 \times 10^2)[\text{urea}] \quad (7)$$

where -7.00×10^3 was obtained in the absence of urea and was independent of $LiClO_4$ concentration *after* the $LiClO_4$-induced transition and 1.22×10^2 is the slope of the curve obtained by adding urea to D($LiClO_4$)-RNase A.

The three unknowns, the fractions of N-, D(urea)-, and D($LiClO_4$)-RNase A, were estimated by fitting the observed to the calculated values of ϵ_{235} and $[\theta]_{220}$ for the mixtures, taking account of the fact that the fractions of each species must sum to unity.

The region in which the fraction of one conformation, e.g., N-RNase A, exceeds the fractions of both of the other two conformations is defined as the one in which N-RNase A is the stable species. The three regions [N-, D(urea)-, and D($LiClO_4$)-RNase A] are separated by curves representing the midpoints of the transition curve between any two species as one denaturant is added at a constant concentration of the other, i.e., the locus of points where the fractions of the two species are equal to each other. These curves (represented by solid lines in Figure 4) were obtained by interpolation because

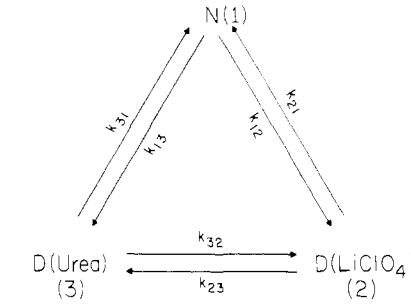

FIGURE 5: Interconversion between N-, D($LiClO_4$)-, and D(urea)-RNase A in the transition region. D($LiClO_4$)-RNase A and D-(urea)-RNase A are represented as single species because each of the folding/unfolding kinetics is represented approximately by a single kinetic constant such as k_{12}, k_{21}, k_{23}, k_{32}, k_{13}, and k_{31}.

the transition curves are not infinitely steep. The three transition curves meet at the triple point where $f_N = f_{D(urea)} = f_{D(LiClO_4)}$. The quantitative aspects of these transition curves differ slightly from, but are close to, those of Ahmad & Bigelow (1979); i.e., they are shifted here to slightly lower concentrations of urea.

Figure 4 shows that $LiClO_4$ (at lower concentrations, <0.4 M) stabilizes the native conformation against urea denaturation. This may be due to a salt effect which reduces electrostatic repulsions among the charged residues. At high concentrations (>0.5 M), $LiClO_4$ behaves as a denaturant. Since both of the transitions (induced by urea and by $LiClO_4$, respectively) can be treated as two state (Creighton, 1979; Ahmad & Bigelow, 1979), only three distinct species, viz., N-, D(urea)-, and D($LiClO_4$)-RNase A, exist in the transition regions (around the solid lines, which meet at the triple point, where all three species are equally stable and are present at the same concentration).

Kinetic Experiments. Since both D(urea)- and D($LiClO_4$)-RNase A each consist of three kinetically distinct species (under folding conditions), one fast-folding species and two slow-folding species (Denton et al., 1982), a manual mixing technique can follow the two slow-folding phases, while the fast-folding phases must be followed with a stopped-flow technique (Denton et al., 1982). In the transition region between native and denatured proteins, the kinetics of folding/unfolding of proteins are approximately single phase (Hagerman & Baldwin, 1976; Nall & Landers, 1981; Goto & Hamaguchi, 1982). In accord with this, we find that the kinetics of interconversion between two species, viz., N- and D($LiClO_4$)-RNase A and N- and D(urea)-RNase A, in the respective transition regions (in the absence of urea and $LiClO_4$, respectively) are single phase; e.g., the folding kinetics from D($LiClO_4$)-RNase A to N-RNase A is expressed by one relaxation time τ (which depends on two rate constants, k_{12} and k_{21}, as in Figure 5), but not by two relaxation times, τ and τ'.

An attempt was made to measure the relaxation time for the interconversion between D($LiClO_4$)-RNase A and D-(urea)-RNase A by using CD since these two species have similar absorption spectra; however, the reaction was too fast to follow with the CD technique. This is reasonable because the formation of an α-helix occurs on a time scale of 10^{-7} s (Cummings & Eyring, 1975; Zana, 1975). Hence, we *assumed* that the interconversion between D($LiClO_4$)-RNase A and D(urea)-RNase A follows single-phase kinetics as observed for the other interconversions described above. In the region of the triple point in Figure 4, these three single-phase kinetic processes proceed simultaneously. Thus, the kinetics of the three-species system shown in Figure 5 can be analyzed

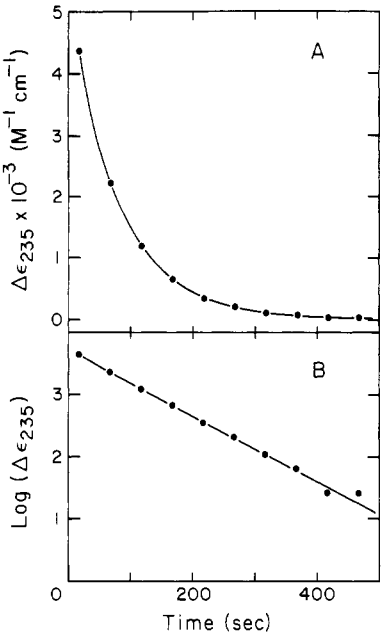

FIGURE 6: Time dependence of (A) $\Delta\epsilon$ and (B) logarithm of $\Delta\epsilon$ at 235 nm of RNase A after change of solvent from 50 mM Gly·HCl (pH 3.0) to 1.30 M $LiClO_4$, 2.24 M urea, and 50 mM Gly·HCl (pH 3.0) at 22 °C. [These final conditions are close to those at the triple point, viz., 1.20 M $LiClO_4$, 2.25 M urea, and 50 mM Gly·HCl (pH 3.0).] The concentration of RNase A decreased from 1.24 mM to 37.3 μM with the change in solvent. The absorbance changes were recorded at 1-s intervals, and some of them are indicated by closed circles in the figure. The solid line shows the computed best least-squares fit to the data by using eq 8 of the text.

(Szabo, 1969), and the absorbance change at 235 nm due to the folding/unfolding of RNase A can be expressed as

$$\Delta\epsilon_{235} = \epsilon_t - \epsilon_\infty = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (8)$$

where ϵ_t and ϵ_∞ are the observed extinction coefficients of the mixture of N-, D(urea)-, and D($LiClO_4$)-RNase A at t seconds and at infinite time, respectively, and A_1 and A_2 are amplitudes of the two phases, while τ_1 and τ_2 are the relaxation time of the two phases. Equation 8 applies at any point in the region around the triple point. The relaxation times, τ_1 and τ_2, are related to the kinetic constants in Figure 5 by

$$1/\tau_{1,2} = \frac{S_1 + S_2}{2} \pm \left[\frac{(S_1 - S_2)^2}{4} + (k_{12} - k_{32})(k_{21} - k_{31}) \right]^{1/2} \quad (9)$$

where $S_1 = k_{12} + k_{31} + k_{13}$ and $S_2 = k_{21} + k_{23} + k_{32}$. In eq 8, ϵ_t and ϵ_∞ are the values obtained after correcting for a very slight linear base-line drift with time. Since τ_1 and τ_2 are independent of the intial concentration of each protein conformation (eq 9), we usually followed the kinetics of *unfolding* of N-RNase A. The reversibility (or independence of τ_1 and τ_2 of the initial conditions) was examined under several solvent conditions; i.e. the same values of τ were also obtained by starting with D(urea)- or D($LiClO_4$)-RNase A. Figure 6 shows the changes of $\Delta\epsilon_{235}$ with time when N-RNase A was mixed with 2.24 M urea and 1.30 M $LiClO_4$. The plot of Log ($\Delta\epsilon_{235}$) vs. time (Figure 6B) clearly shows that there are one major and one minor phase (the minor phase being indicated by slight curvature in the line). The relaxation times of the major phase under various final solvent conditions are plotted in Figure 4, and similar values of the relaxation times are connected by dashed lines. It should be noted that all of the experimental data were obtained under solution conditions that differ from those at the triple point; the data were then extrapolated to the triple point (see below).

The relaxation time of the folding/unfolding reaction is long in the region where N-RNase A is the most stable species. As the concentration of denaturant is increased, i.e., as the system passes through the transition region from native to denatured species, the relaxation time is seen to be reduced markedly, as also observed by Hagerman & Baldwin (1976). The relaxation time at the triple point was obtained by extrapolation of the data from many kinetic experiments *around* the triple point, and found to be 90 s.

It is difficult to estimate kinetic constants from eq 8 and 9 because of their complex form. However, we can do so by the following argument. At low concentrations of urea, D-(urea)-RNase A is unstable and contributes negligibly to the interconversion between N-RNase A and D($LiClO_4$)-RNase A (lower portion of Figure 4). Thus, the kinetics of the interconversion between N-RNase A and D($LiClO_4$)-RNase A at low concentrations of urea can be treated as a two-species, single-phase system (Szabo, 1969). The equations corresponding to eq 8 and 9 are

$$\epsilon_t - \epsilon_0 = Ae^{-t/\tau} \quad (10)$$

$$\tau = 1/(k_{12} + k_{21}) \quad (11)$$

The relaxation time, τ, was measured in the transition region between N-RNase A and D($LiClO_4$)-RNase A at low concentrations of urea (<1 M urea). Then, the values of τ were extrapolated to the transition point (lower part of solid line in Figure 4) between N-RNase A and D($LiClO_4$)-RNase A at various *low* concentrations of urea. These values of τ were found to be 100 s and independent of the concentration of urea in the range [urea] < 1 M. Since $k_{12} = k_{21}$ at the transition point, and these values are independent of urea concentration, we assumed that they could be extrapolated along the transition line between N-RNase A and D($LiClO_4$)-RNase A to the triple point (2.25 M urea). This extrapolation yielded a value of 0.005 s^{-1} for k_{12} and k_{21} at the triple point.

In a similar way, the kinetics for the interconversion between N-RNase A and D(urea)-RNase A were measured at low concentrations of $LiClO_4$ (<0.5 M $LiClO_4$), where D($LiClO_4$)-RNase A is unstable and can be ignored. The relaxation time in the transition region between N-RNase A and D(urea)-RNase A was found to be 200 s and independent of the concentration of $LiClO_4$ in the range [$LiClO_4$] < 0.5 M. Extrapolating to the triple point ([$LiClO_4$] = 1.20 M) yielded $k_{13} = k_{31} = 0.0025$ s^{-1}.

The validity of these extrapolated values was checked by the following analysis of the kinetics of the three-species system. First of all, since the interconversion between D-($LiClO_4$)-RNase A and D(urea)-RNase A is too fast to follow by the CD technique, we assumed a very large value for $k_{23} = k_{32}$ at the triple point; any value >0.5 s^{-1} suffices. Then the extrapolated values of the six kinetic constants at the triple point were inserted in eq 9, giving 90 and <1 s for the relaxation times of the major and minor phases, respectively [with amplitudes of 5.3×10^3 M^{-1} cm^{-1} and the negligibly small value of <0.03 M^{-1} cm^{-1}, respectively, computed from eq 1–3 of Szabo (1969)]. The agreement between this computed value of 90 s for the major phase and the observed relaxation time of 90 s for the folding/unfolding kinetics at the triple point (Figure 4) supports the validity of the above extrapolations.

The small difference in the values of τ between N-RNase A and D($LiClO_4$)-RNase A and between N-RNase A and D(urea)-RNase A (100 vs. 200 s which corresponds to only 400 cal/mol in activation free energy, if the same preexpo-

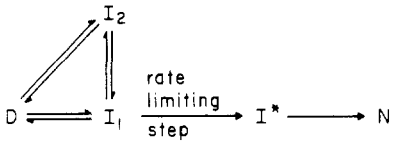

FIGURE 7: Schematic representation of folding pathway. D is the starting denatured protein, I_1 and I_2 are "Intermediates", the reaction from I_1 to I* is the rate-limiting step, I* is an "Intermediate*", and N is the refolded protein.

nential factor is assumed) demonstrates that the α-helices and the compact structure in D($LiClO_4$)-RNase A are not functioning as prenucleation or nucleation site(s); i.e., there is no significant acceleration of folding from D($LiClO_4$)-RNase compared to D(urea)-RNase A.

Discussion

The roles played by ordered structures in folding pathways of proteins have been examined in two ways. In the first way, attention has been focused on detecting ordered structures in the "Intermediates", and it has been shown by CD measurements, (Denton et al., 1982; Goto & Hamaguchi, 1982; McPhie, 1982), by NMR measurements (Blum et al., 1978; Birringer & Fink, 1982), and by amide proton exchange experiments (Schmid & Baldwin, 1979; Kim & Baldwin, 1980) that kinetically trapped "Intermediates" in the folding of proteins with intact disulfide bonds involve ordered structures to some extent. This demonstrates that some residues or fragments have intrinsic tendencies to form ordered structures before the entire molecule folds, supporting the idea that short- and medium-range interactions control the early stages of folding (Tanaka & Scheraga, 1975, 1977; Wako & Saitô, 1978; Lim, 1980; Gō & Abe, 1981). However, this does not necessarily mean that these ordered structures contribute to the folding pathways. This is illustrated in the scheme of Figure 7. Even though ordered structures may exist in I_2 (but not in D and I_1) they do not contribute to the pathway because such ordered structures in I_2 can be destroyed in the further steps of the pathway. A typical example of such ordered structures was reported for the regeneration of bovine pancreatic trypsin inhibitor from its reduced form (Creighton, 1978). An immunological assay (Creighton et al., 1978), difference absorption spectra (Kosen et al., 1980), and CD spectra (Kosen et al., 1983) showed that an "Intermediate" with two *native* disulfide bonds (between Cys-31 and Cys-51, and between Cys-14 and Cys-38) has more ordered nativelike conformations than other "Intermediates". However, these *nativelike ordered* structures have to be disrupted during the reshuffling of the native disulfide bonds to form a nonnative disulfide bond in order to regenerate the protein. Furthermore, this nativelike "Intermediate" is not essential on the pathway, even though it is populated in the kinetic process of regeneration.

In a second way, attention has been focused on the rate-limiting step of the kinetic folding. As described in the introduction, ordered structures can contribute in the kinetic folding by forming prenucleation sites, nucleation sites, or "Intermediates*". This paper and that of Denton et al. (1982) have examined the roles of an α-helix and of compactness of RNase A induced by $LiClO_4$. A compact structure has been considered to be a characteristic of the native conformation of a protein, and the concept of compactness is used in packing models to predict the three-dimensional structures of proteins (Ptitsyn & Rashin, 1975; Lim & Efimov, 1977; Efimov, 1979; Cohen et al., 1982) and in distance–constraint prediction methods (Kuntz et al., 1979; Ycas et al., 1978; Goel & Ycas, 1979). A similar compact structure with an α-helix has been reported to exist in a folding intermediate of α-lactalbumin, and on the basis of equilibrium studies, this intermediate is thought to lie on the folding pathway (Kuwajima, 1977; Dolgikh et al., 1981). However, this paper and that of Denton et al. (1982) demonstrate that the compactness and the presence of an α-helix do not contribute to the kinetic folding of RNase A. This suggests that short- and medium-range interactions (which give rise to such ordered structures) may not function to produce prenucleation or nucleation sites, and long-range interactions may possibly be required to form such sites. For example, acceleration of some of the slow-folding kinetics of RNase A by addition of ammonium sulfate (Cook et al., 1979; Lin & Brandts, 1983), which binds to the active site of the protein, suggests that the formation of the active site may occur in the rate-limiting step. The refolding of the active site requires the association of His-12, Lys-41, and His-119 by long-range interactions. Thus, long-range interactions may function in the rate-limiting steps in some folding pathways.

Conclusion

The compact structure and α-helix have little effect on the kinetics of refolding of RNase A. This demonstrates that even though the protein becomes compact and forms α-helices during the early stages of the folding pathway(s), these are not functioning as prenucleation or nucleation site(s). Possibly long-range interactions are required to establish the prenucleation and nucleation sites.

Acknowledgments

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Registry No. $LiClO_4$, 7791-03-9; RNase A, 9001-99-4.

References

Acharya, A. S., & Taniuchi, H. (1982) *Mol. Cell. Biochem. 44*, 129.
Ahmad, F., & Bigelow, C. C. (1979) *J. Mol. Biol. 131*, 607.
Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.
Bierzynski, A., & Baldwin, R. L. (1982) *J. Mol. Biol. 162*, 173.
Biringer, R. G., & Fink, A. L. (1982) *Biochemistry 21*, 4748.
Blum, A. D., Smallcombe, S. H., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 305.
Borkakoti, N. (1983) *Eur. J. Biochem. 132*, 89.
Borkakoti, N., Moss, D. S., & Palmer, R. A. (1982) *Acta Crystallogr., Sect. B 38*, 2210.
Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350.
Cohen, F. E., Sternberg, J. E., & Taylor, W. R. (1982) *J. Mol. Biol. 156*, 821.
Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157.
Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231.
Creighton, T. E. (1979) *J. Mol. Biol. 129*, 235.
Creighton, T. E., Kalef, E., & Arnon, R. (1978) *J. Mol. Biol. 123*, 129.
Cummings, A. L., & Eyring, E. M. (1975) *Biopolymers 14*, 2107.
Denton, J. B., Konishi, Y., & Scheraga, H. A. (1982) *Biochemistry 21*, 5155.
Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Yu., &

Ptitsyn, O. B. (1981) *FEBS Lett. 136*, 311.
Efimov, A. V. (1979) *J. Mol. Biol. 134*, 23.
Finkelstein, A. V., & Ptitsyn, O. B. (1971) *J. Mol. Biol. 62*, 613.
Gō, N., & Abe, H. (1981) *Biopolymers 20*, 991.
Goel, N. S., & Ycas, M. (1979) *J. Theor. Biol. 77*, 253.
Goto, Y., & Hamaguchi, K. (1982) *J. Mol. Biol. 156*, 891.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108.
Hagerman, P. J., & Baldwin, R. L. (1976) *Biochemistry 15*, 1462.
Kim, P. S., & Baldwin, R. L. (1980) *Biochemistry 19*, 6124.
Klee, W. A. (1968) *Biochemistry 7*, 2731.
Konishi, Y., Ooi, T., & Scheraga, H. A. (1981) *Biochemistry 20*, 3945.
Konishi, Y., Ooi, T., & Scheraga, H. A. (1982a) *Biochemistry 21*, 4734.
Konishi, Y., Ooi, T., & Scheraga, H. A. (1982b) *Biochemistry 21*, 4741.
Konishi, Y., Ooi, T., & Scheraga, H. A. (1982c) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5734.
Kosen, P. A., Creighton, T. E., & Blout, E. R. (1980) *Biochemistry 19*, 4936.
Kosen, P. A., Creighton, T. E., & Blout, E. R. (1983) *Biochemistry 22*, 2433.
Kotelchuck, D., & Scheraga, H. A. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 1163.
Kuntz, I. D., Crippen, G. M., & Kollman, P. A. (1979) *Biopolymers 18*, 939.
Kuwajima, K. (1977) *J. Mol. Biol. 114*, 241.
Labhardt, A. M. (1982) *J. Mol. Biol. 157*, 331.
Labhardt, A. M., & Baldwin, R. L. (1979a) *J. Mol. Biol. 135*, 231.
Labhardt, A. M., & Baldwin, R. L. (1979b) *J. Mol. Biol. 135*, 245.
Labhardt, A. M., Ridge, J. A., Lindquist, R. N., & Baldwin, R. L. (1983) *Biochemistry 22*, 321.
Lim, V. I. (1980) in *Protein Folding* (Jaenicke, R., Ed.) p 149, Elsevier/North-Holland Biomedical Press, Amsterdam.
Lim, V. I., & Efimov, A. V. (1977) *FEBS Lett. 78*, 279.
Lin, L.-N., & Brandts, J. F. (1983) *Biochemistry 22*, 564.
McPhie, P. (1982) *Biochemistry 21*, 5509.
Miyazawa, S., & Jernigan, R. L. (1982) *Biochemistry 21*, 5203.
Nall, B. T., & Landers, T. A. (1981) *Biochemistry 20*, 5403.
Némethy, G., & Scheraga, H. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6050.
Ptitsyn, O. B., & Rashin, A. A. (1975) *Biophys. Chem. 3*, 1.
Scheraga, H. A. (1973) *Pure Appl. Chem. 36*, 1.
Schmid, F. X., & Baldwin, R. L. (1979) *J. Mol. Biol. 135*, 199.
Sela, M., & Anfinsen, C. B. (1957) *Biochim. Biophys. Acta 24*, 229.
Silverman, D. N., Kotelchuck, D., Taylor, G. T., & Scheraga, H. A. (1972) *Arch. Biochem. Biophys. 150*, 757.
Szabo, Z. G. (1969) *Compr. Chem. Kinet. 2*, 1.
Tamburro, A. M., Scatturin, A., Rocchi, R., Marchiori, F., Borin, G., & Scoffone, E. (1968) *FEBS Lett. 1*, 298.
Tanaka, S., & Scheraga, H. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3802.
Tanaka, S., & Scheraga, H. A. (1977) *Macromolecules 10*, 291.
Wako, H., & Saitô, N. (1978) *J. Phys. Soc. Jpn. 44*, 1931.
Wlodawer, A., & Sjölin, L. (1983) *Biochemistry 22*, 2720.
Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., & Richards, F. M. (1970) *J. Biol. Chem. 245*, 305.
Ycas, M., Goel, N. S., & Jacobsen, J. W. (1978) *J. Theor. Biol. 72*, 443.
Zana, R. (1975) *Biopolymers 14*, 2425.