Influence of an Extrinsic Cross-Link on the Folding Pathway of Ribonuclease A. Kinetics of Folding–Unfolding†

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ABSTRACT: The kinetics of folding/unfolding of cross-linked Lys^7-dinitrophenylene-Lys^{41}-ribonuclease A were studied and compared to those of unmodified ribonuclease A (RNase A) at various concentrations of guanidine hydrochloride. The folding of the denatured cross-linked protein involved one fast-folding species (22 ± 4%) and two slow-folding species, as observed in unmodified ribonuclease A. Also, a nativelike intermediate, analogous to that reported previously for unmodified ribonuclease A [Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157], has been detected on the folding pathway of cross-linked ribonuclease A. The extrinsic cross-link between Lys^7 and Lys^{41} did not affect the rate constants for the folding kinetics of these three species. The cross-link did, however, significantly affect the rate constant for unfolding of the native protein. The conformation of the protein in the transition state of the unfolding pathway was deduced from an analysis of the kinetic data. It appears that the 41 N-terminal residues are unfolded in the transition state of the unfolding pathway. Thus, the unfolding pathway of RNase A is *sequential* in that further unfolding (after the transition state) follows the unfolding of the 41 N-terminal residues. Also, the *conformation* of the 41 N-terminal residues does not play a role in the folding pathway. Presumably, if the cross-link were introduced instead between two *other* residues that are in the segment(s) involved in the rate-limiting step(s), it could increase the refolding rate constants and possibly the concentration of fast-folding species.

The various roles of the amino acid residues in proteins have been studied extensively. Some residues are important for the biological activity or for the stability of the native conformation, while others may play significant roles in protein folding. This paper is concerned with the latter role. Denton et al. (1982) and Lynn et al. (1984) have shown that different amino acid residues or ordered structures in ribonuclease A (RNase A)[1] have distinct effects on the folding pathway. Thus, it is of interest to identify the amino acid residues or conformations that are essential/nonessential for the folding of this protein.

One approach to identify the essential/nonessential residues in protein folding is either to stabilize or to destabilize conformations in a particular portion of the chain and then to measure the resulting effects on refolding/unfolding rates. With regard to thermodynamic stability, some intramolecular cross-links do not affect the native conformation of a protein; instead, they destabilize the denatured conformations by decreasing the chain entropy of the flexible denatured conformations (Anfinsen & Scheraga, 1975; Johnson et al., 1978; Lin et al., 1984). If such a cross-link encourages interactions required in the transition state for folding, then the rate of folding will be increased. Conversely, if the cross-link favors interactions which must be disrupted in the transition state, then the rate of unfolding will be decreased. For example, the constant fragment of the immunoglobulin light chain has one intramolecular disulfide bond and retains the stable native conformation in its reduced form. Goto & Hamaguchi (1982a,b) studied the effect of the disulfide bond on the folding kinetics and observed a significant acceleration due to the presence of the disulfide bond. Presumably, the disulfide loop segment is folded in the transition state. The role of an extrinsic cross-link in the regeneration kinetics from reduced and cross-linked RNase A (between Lys^7 and Lys^{37} or Lys^{31} and Lys^{37}) was studied by Takahashi & Ooi (1973), who found no enhancement of the regeneration rate in comparison with the unmodified protein. Segawa (1984) measured the folding/unfolding kinetics of cross-linked lysozyme (between Glu^{35} and Trp^{108}) using 4.5 M LiBr or 4.6 M propanol as a denaturant. The effect of the cross-link depended on the denaturant; i.e., a significant enhancement of the folding rate constant was observed in LiBr but not in propanol, and a significant decrease of the unfolding rate constant was observed in propanol but not in LiBr. This indicates that the structures in the transition states in the folding/unfolding pathways of the cross-linked lysozyme differ in LiBr and in propanol and that the effect of the cross-link depends on the pathway. Thus, the folding/unfolding pathways and the location of the cross-link are critical factors in determining the effect of a cross-link on the kinetics.

We have used a cross-link between Lys^7 and Lys^{41} in RNase A (Lin et al., 1984) for the following reasons. Sulfate ion is known to interact with His^{12}, Lys^{41}, and His^{119} in the active site of native RNase A (Borkakoti et al., 1982) and to increase the thermal stability of the native conformation (von Hippel & Wong, 1965; Ginsburg & Carroll, 1965). If all or part of His^{12}, Lys^{41}, and His^{119} are associated in the transition state

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; CL(7–41)-RNase A, cross-linked derivative of RNase A (Lys^7-dinitrophenylene-Lys^{41}-RNase A); RNase S, derivative of RNase A in which the peptide bond between residues 20 and 21 has been cleaved; S protein, residues 21–124 of RNase S; S peptide, residues 1–20 of RNase S; Gdn·HCl, guanidine hydrochloride; Tris·HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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of the folding pathway, sulfate might be expected to accelerate the folding kinetics by binding to and stabilizing the transition state. An acceleration by ammonium sulfate was observed in the major slow phase of RNase A folding, and no acceleration occurred in the fast and minor slow phases (Lin & Brandts, 1983). Here, we have studied the effect on the folding/unfolding kinetics of the forced association between the N-terminal α-helix, which involves His^{12}, and the portion of the chain around Lys^{41} by using a cross-link between Lys^{7} and Lys^{41}.

Experimental Procedures

Materials. The materials used in this paper were the same as those described in Lin et al. (1984). In addition, sodium cacodylate was obtained from Sigma Chemical Co., and $HClO_4$ was reagent grade from Fisher Scientific.

Equilibrium Measurements. Solutions of unmodified RNase A or CL(7–41)-RNase A (both at 73 μM) in 50 mM Gly·HCl (pH 2.0) or in 50 mM sodium cacodylate (pH 5.8) with various concentrations of Gdn·HCl were prepared. The concentrations of unmodified RNase A and CL(7–41)-RNase A were determined spectrophotometrically at 22 °C by using $\epsilon_{275} = 9300\ M^{-1}\ cm^{-1}$ and $\epsilon_{275} = 11\,490\ M^{-1}\ cm^{-1}$, respectively, where 275 nm is the absorption isosbestic point between the native and Gdn·HCl-denatured forms for both unmodified RNase A and CL(7–41)-RNase A (Lin et al., 1984). The concentrations of Gdn·HCl were determined from the refractive index of the solutions (Nozaki, 1972) with an Abbé refractometer (Bausch & Lamb Optical Co). After the protein solution reached equilibrium (by incubation for about 4 h), the absorbances of the solution at 275 and 287 nm were measured by using a modified Cary Model 14 spectrophotometer (Denton et al., 1982).

Stopped-Flow Measurements. The effect of the extrinsic cross-link on the kinetics of folding and unfolding of RNase A was studied by stopped-flow measurements using a Durrum D-100 stopped-flow apparatus with an optical system described elsewhere (Nall & Landers, 1981). The absorbance change was monitored at 287 nm, and the drive syringes and observation chamber were thermostated at 45.0 ± 0.1 °C. The refolding kinetics at pH 5.8 were obtained by using 1:5 mixing. Unmodified RNase A (730 μM) or CL(7–41)-RNase A (730 μM) in 3 M Gdn·HCl at pH 2.0 (adjusted with 1.5 M $HClO_4$) was placed in the lower drive syringe while 0.10 M sodium cacodylate (pH ~6) was placed in the upper drive syringe in order to avoid flow artifacts caused by premixing. Thus, the final folding conditions were 122 μM protein in 0.5 M Gdn·HCl and 0.08 M sodium cacodylate (pH 5.8 ± 0.1) at 45 °C. The kinetic traces were stored in a Biomation Model 805 transient recorder, transferred to an Apple II+ computer, and saved as disk files.

The unfolding kinetics of unmodified RNase A at pH 5.8 were obtained by mixing 5 volumes of 7.2 M Gdn·HCl in 50 mM sodium cacodylate (pH 5.8) with 1 volume of 720 μM unmodified RNase A in 50 μM sodium cacodylate (pH 5.8) at 25.0 °C. The final unfolding conditions were 120 μM protein in 6.0 M Gdn·HCl in 0.05 M sodium cacodylate (pH 5.8) at 25 °C.

The digitized kinetic data were analyzed by using the following empirical equation for fitting with two time constants, τ_1 and τ_2 (Denton et al., 1982):

$$A_\infty - A_t = B_1 e^{-t/\tau_1} + B_2 e^{-t/\tau_2} \quad (1)$$

where A_∞ and A_t are the relative absorbances at infinite time and at t seconds, respectively. B_1 and τ_1 are the amplitude and time constant, respectively, of the first phase, while B_2 and τ_2 are the amplitude and time constant, respectively, of the second phase.

Manual Mixing Experiments. To obtain more precise data for the slow phase under the same folding conditions used in the stopped-flow experiments, the slow-folding kinetics of unmodified RNase A and CL(7–41)-RNase A were also measured by manual mixing experiments, using an HP 8450A UV–vis spectrophotometer.

The temperature dependence of the refolding kinetics of the slow phases was obtained manually by mixing 40 μL of 3.65 mM unmodified RNase A or CL(7–41)-RNase A in 50 mM Gly·HCl (pH 2.0) with 1.96 mL of 50 mM sodium cacodylate (pH 5.8) in the temperature range of 1–20 °C. The final solution was 73 μM in unmodified RNase A or Cl(7–41)-RNase A and 50 mM in sodium cacodylate (pH 5.8). The temperature, controlled with a Forma-Temp Jr. bath, was measured with a calibrated dipping thermistor to an accuracy of ±0.1 °C.

Similarly, the effects of various concentrations of Gdn·HCl on the slow-unfolding kinetics or on the slow-refolding kinetics of unmodified RNase A and CL(7–41)-RNase A were also studied by manual mixing experiments at 22 °C. After mixing for 15 s, the folding/unfolding kinetics were followed by recording the change of absorbance at 287 nm for about 6 times longer than the relaxation time of the process. The data were collected for 1-s periods, averaged with an analogue to digital converter connected to the modified Cary Model 14 spectrophotometer, and then sent directly to a Prime 550 computer for a kinetic analysis using eq 1.

Reversibility of Unfolding/Refolding in Equilibrium or in Kinetics. The reversibility of Gdn·HCl unfolding and refolding in the equilibrium and in the kinetic studies was examined by column chromatography. Unmodified RNase A or CL(7–41)-RNase A in 6 M Gdn·HCl (pH 2.0 or 5.8) was loaded on a gel filtration P-6DG column (2.5 × 30 cm, equilibrated with 0.1 N acetic acid) in order to remove Gdn·HCl and refold the proteins, followed by lyophilization. The refolded unmodified RNase A or CL(7–41)-RNase A from the kinetic experiments (Figures 3 and 5) was also loaded on the same gel column to remove small amounts of Gdn·HCl and was lyophilized. Refolded unmodified RNase A or CL(7–41)-RNase A was mixed with equal amounts of unmodified RNase A or CL(7–41)-RNase A, respectively, in the native conformation in 5 mM Tris·HCl (pH 8.0). The protein solutions were fractionated on a carboxymethylcellulose column (1.1 × 30 cm, pH 8.0) with a 0–0.11 M NaCl linear gradient formed by mixing 250 mL each of 5 mM Tris·HCl (pH 8.0) and 0.11 M NaCl in 5 mM Tris·HCl (pH 8.0). The flow rate was 0.46 mL/min, and the fractionation was monitored by the absorbance at 280 nm.

Detection of a Nativelike Intermediate. The procedures of Schmid (1983) were followed to test for the existence and stability of a nativelike intermediate with the following modifications. Unmodified RNase A or CL(7–41)-RNase A (7.30 mM) was denatured in 6 M Gdn·HCl and 50 mM Gly·HCl (pH 2.0) for 2 h at room temperature, and then the temperature was adjusted to 0 °C. Prefolding to accumulate the nativelike intermediate was achieved by mixing 10 μL of the denatured protein solution (7.30 mM) with 190 μL of 50 mM sodium cacodylate (pH 5.8) containing 0.8 M $(NH_4)_2SO_4$ at 0 °C for 15 s. The stability of the nativelike intermediate was assayed immediately by pipetting 1.8 mL of 50 mM sodium cacodylate (pH 5.8) containing 0.3, 3, or 4 M Gdn·HCl (for unmodified RNase A) or containing 0.3, 4.8, or 5.8 M Gdn·HCl [for CL(7–41)-RNase A] into 200 μL of the protein

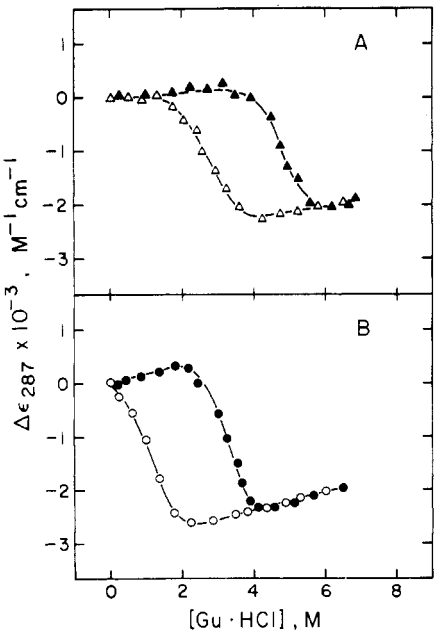

FIGURE 1: Equilibrium Gdn·HCl-induced denaturation, monitored by the absorbance change at 287 nm, of (A) CL(7–41)-RNase A (73 μM) at pH 2.0 (△) and 5.8 (▲) and of (B) unmodified RNase A (73 μM) at pH 2.0 (○) and 5.8 (●) at 22 °C. Buffers used were 50 mM Gly·HCl for pH 2.0 and 50 mM sodium cacodylate for pH 5.8, respectively.

solution and following the absorbance change at 287 nm and at 10 °C. The final concentration of the protein was 36.5 μM.

Results

Equilibrium Transition. Lin et al. (1984) showed that the extrinsic cross-link between Lys^7 and Lys^{41} in RNase A increases the thermal stability of the native conformation relative to the denatured ones by 4.9 kcal/mol at 40 °C and pH 2.0. Similarly, the presence of this cross-link stabilizes the native conformation of RNase A against Gdn·HCl denaturation. Figure 1 shows the Gdn·HCl-induced equilibrium conformational transitions of unmodified RNase A and CL(7–41)-RNase A at pH 2.0 and 5.8 at 22 °C.

The reversibility of the Gdn·HCl denaturation was examined by column chromatography. The results showed that refolded unmodified RNase A or CL(7–41)-RNase A coeluted from the ion-exchange column as a single peak with unmodified RNase A or CL(7–41)-RNase A, respectively, in the native conformation. No irreversibly denatured protein was detected in the chromatograms. Thus, we conclude that Gdn·HCl unfolding/refolding of unmodified RNase A and CL(7–41)-RNase A at pH 2.0 (or 5.8) and 22 °C is reversible.

The apparent equilibrium constant, K_{app}, for the transition between the native and Gdn·HCl-denatured states can be written as

$$K_{app} = \frac{(\Delta OD_{287})_N[\text{Gdn·HCl}] - \Delta OD_{287}[\text{Gdn·HCl}]}{\Delta OD_{287}[\text{Gdn·HCl}] - (\Delta OD_{287})_D[\text{Gdn·HCl}]} = e^{-(\Delta G^\circ_{app}/RT)} \quad (2)$$

where ΔG°_{app} is the apparent free energy difference between the native and Gdn·HCl-denatured states, $(\Delta OD_{287})_N$[Gdn·HCl] and $(\Delta OD_{287})_D$[Gdn·HCl] are ΔOD_{287} values of the native and Gdn·HCl-denatured states, respectively, extrapolated to the given Gdn·HCl concentration, and ΔOD_{287}[Gdn·HCl] is the measured value of ΔOD_{287} at the given Gdn·HCl concentration. Within a range of experimental error, the difference in the apparent free energy of unfolding of these two proteins was insensitive to the Gdn·HCl concentration and found to be 3.0 kcal/mol at pH 5.8, where the refolding kinetics were measured.

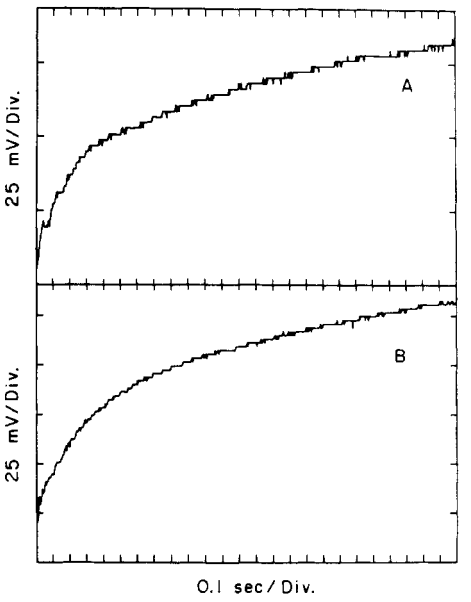

FIGURE 2: Stopped-flow measurements of refolding kinetics of CL-(7–41)-RNase A (A) and unmodified RNase A (B) monitored by the absorbance change at 287 nm. A solution of 7.3×10^{-4} M unmodified RNase A or CL(7–41)-RNase A in 3.0 M Gdn·HCl (pH 2.0) was mixed with 5 times the volume of 0.10 M sodium cacodylate (pH 5.8) at 45 °C. The final solution conditions were 1.22×10^{-4} M unmodified RNase A or CL(7–41)-RNase A in 0.5 M Gdn·HCl and 0.08 M sodium cacodylate (pH 5.8) at 45 °C.

Similar equilibrium measurements showed that 3.0 M Gdn·HCl (pH 2.0) sufficiently denatures both the unmodified RNase A and CL(7–41)-RNase A at 45 °C, whereas both of these proteins are in the native conformation in 0.5 M Gdn·HCl (pH 5.8) at 45 °C. Thus, these two solvent conditions were used in the following stopped-flow kinetic folding experiments.

Kinetic Folding Experiments. Gdn·HCl-denatured RNase A refolds kinetically through three different pathways corresponding to the fast, major slow, and minor slow phases when the Gdn·HCl concentration is rapidly lowered by a solvent jump (Cook et al., 1979). The effect of the extrinsic cross-link between Lys^7 and Lys^{41} on these three phases was examined. Figure 2 shows the refolding kinetics of CL(7–41)-RNase A (Figure 2A) and of unmodified RNase A (Figure 2B) using a stopped-flow apparatus when the denaturing solvent [3.0 M Gdn·HCl (pH 2.0)] is jumped to the folding solvent [0.5 M Gdn·HCl and 0.08 M sodium cacodylate (pH 5.8)] at 45 °C. Under these final conditions, the major and minor slow phases combine to give a single phase in the refolding of both unmodified RNase A and CL(7–41)-RNase A. Merging of the two slow phases into one has also been observed in the refolding of urea-denatured RNase A (Lin & Brandts, 1983). Thus, the kinetic data in Figure 2 were analyzed with two relaxation times corresponding to the fast and slow phases (eq 1). For the slow phase, improved precision was obtained in manual mixing experiments under the same folding conditions. The resulting parameters (amplitude and relaxation time) were used to determine the base line for the fast phase (Figure 2). The relative amplitudes of the absorbance change at 287 nm for the fast and slow phases of unmodified RNase A and CL(7–41)-RNase A were estimated from panels B and A, respectively, of Figure 2, giving 20% ± 2% and 22% ± 4% for the fast phase, respectively. Garel et al. (1976) reported a 20% fast phase for unmodified RNase A. Thus, the extrinsic cross-link does not stabilize the fast-folding species in the

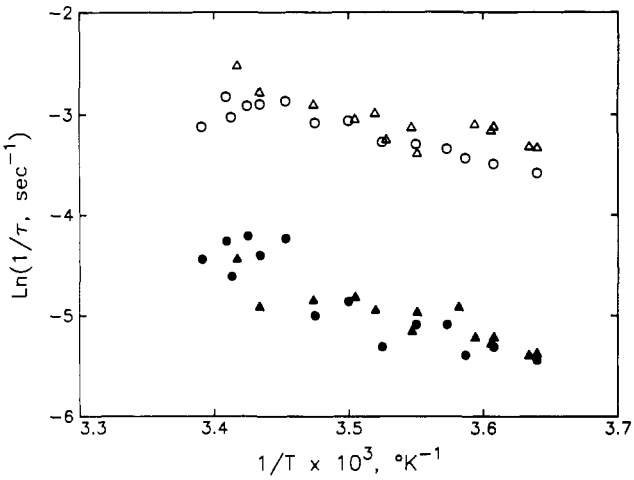

FIGURE 3: Arrhenius plots describing the temperature dependence of the refolding relaxation times of the major (△) and minor (▲) slow phases of CL(7–41)-RNase A and of the major (○) and minor (●) slow phases of unmodified RNase A. CL(7–41)-RNase A (3.65×10^{-3} M) or unmodified RNase A (3.65×10^{-3} M) in 6 M Gdn·HCl and 50 mM Gly·HCl (pH 2.0) was diluted 50 times by a manual mixing technique at various temperatures (2–22 °C). The final solution conditions were 7.3×10^{-5} M CL(7–41)-RNase A or unmodified RNase A in 0.12 M Gdn·HCl and 50 mM sodium cacodylate (pH 5.8).

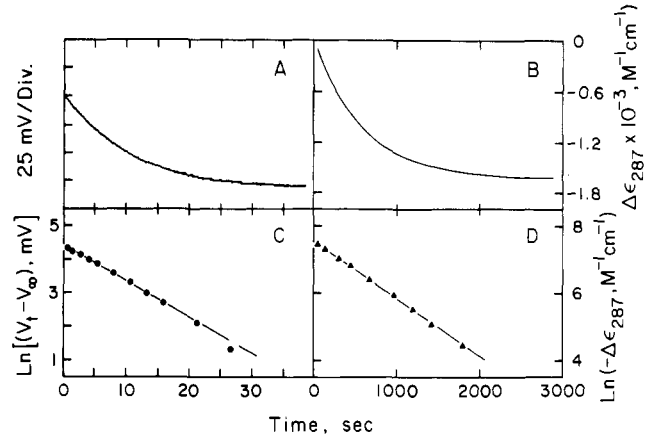

FIGURE 4: Unfolding kinetics of unmodified RNase A measured by stopped flow (A) and of CL(7–41)-RNase A measured by manual mixing (B). (C) and (D) are semilog plots of the absorbance change at 287 nm pertaining to traces in (A) and (B), respectively. The closed circles and triangles are some of the experimental data, and the connecting solid lines are the results of least-squares curve fitting. Initial conditions were 7.2×10^{-4} M unmodified RNase A or 3.65×10^{-3} M CL(7–41)-RNase A in 50 mM sodium cacodylate (pH 5.8) at 25 °C. Final conditions were 1.2×10^{-4} M unmodified RNase A or 7.3×10^{-5} M CL(7–41)-RNase A in 6 M Gdn·HCl and 50 mM sodium cacodylate (pH 5.8) at 25 °C.

denatured conformation of RNase A. The relaxation times were 0.39 ± 0.09 and 14.0 ± 0.7 s for unmodified RNase A and 0.35 ± 0.16 and 14 ± 1 s for CL(7–41)-RNase A, demonstrating that the extrinsic cross-link between Lys^7 and Lys^{41} does not affect the refolding kinetic constants for the fast and two slow phases of RNase A in 0.5 M Gdn·HCl at pH 5.8 and 45 °C.

Since the major and minor slow-folding phases show distinctive relaxation times in 0.12 M Gdn·HCl at pH 5.8 and 2–22 °C, the relaxation times of these two slow-folding phases were measured under the following conditions by a manual mixing technique, where the fast phase was completed within the mixing time. Forty microliters of unmodified RNase A or CL(7–41)-RNase A (3.65 mM) in 6 M Gdn·HCl and 50 mM Gly·HCl (pH 2.0) was mixed with 1.96 mL of 50 mM sodium cacodylate (pH 5.8). The final solution contained protein at a concentration of 73 μM, 0.12 M Gdn·HCl, and 50 mM sodium cacodylate (pH 5.8). The refolding kinetics were measured at various temperatures (Figure 3). The relaxation times of unmodified RNase A and CL(7–41)-RNase A were in good agreement over the temperature range of 2–22 °C. The relative amplitudes of the absorbance change at 287 nm due to the major and minor slow-folding phases were also in good agreement, viz., 80% ± 5% and 20% ± 5%, respectively, for both unmodified RNase A and CL(7–41)-RNase A. This demonstrates that the extrinsic cross-link between Lys^7 and Lys^{41} affects neither the refolding rate constants nor the activation energies of the major and minor slow-folding phases.

Kinetic Unfolding Experiments. The unfolding kinetics were measured for unmodified RNase A by the stopped-flow technique (Figure 4A) and for CL(7–41)-RNase A by the manual mixing technique (Figure 4B). One volume of unmodified RNase A (0.72 mM) in 50 mM sodium cacodylate (pH 5.8) was mixed with 5 volumes of 7.2 M Gdn·HCl and 50 mM sodium cacodylate (pH 5.8) at 25 °C. The final solution was 0.12 mM unmodified RNase A, 6 M Gdn·HCl, and 50 mM sodium cacodylate (pH 5.8). Figure 4C plots the log of the absorbance change at 287 nm, demonstrating that the unfolding kinetics occur in a single phase with a relaxation

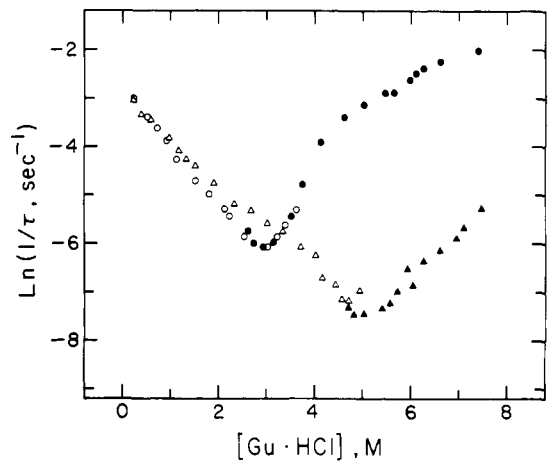

FIGURE 5: Effect of the concentration of Gdn·HCl on the kinetic relaxation times of CL(7–41)-RNase A [*major* phases of refolding (△) and unfolding (▲)] and of unmodified RNase A [*major* phases of refolding (○) and unfolding (●)] monitored by the absorbance change at 287 nm and analyzed with eq 1. Initial conditions for refolding were 3.65×10^{-3} M protein in 6 M Gdn·HCl and 50 mM Gly·HCl (pH 2.0) and for unfolding were 3.65×10^{-3} M protein in 50 mM sodium cacodylate (pH 5.8). All final conditions were 7.3×10^{-5} M protein at the indicated concentrations of Gdn·HCl and 50 mM sodium cacodylate (pH 5.8) at 22 °C.

time of 9.0 s. The unfolding kinetics of CL(7–41)-RNase A in 6 M Gdn·HCl and 50 mM sodium cacodylate (pH 5.8) were so slow that the process was followed by the manual mixing technique (Figure 4B). Figure 4D plots the log of the absorbance change at 287 nm, demonstrating that the unfolding kinetics occur in a single phase with a relaxation time of 580 s. Thus, the extrinsic cross-link between Lys^7 and Lys^{41} strongly retards unfolding but does not affect the rate constants for folding.

The effect of the extrinsic cross-link on the folding/unfolding kinetics was studied further at various concentrations of Gdn·HCl at pH 5.8 and 22 °C. Figure 5 shows the relaxation times of the *major* phase in the folding/unfolding kinetic measurements of unmodified RNase A and CL(7–41)-RNase A. Below 3 M Gdn·HCl, the relaxation time for the major kinetic phase in folding/unfolding is the same for both proteins

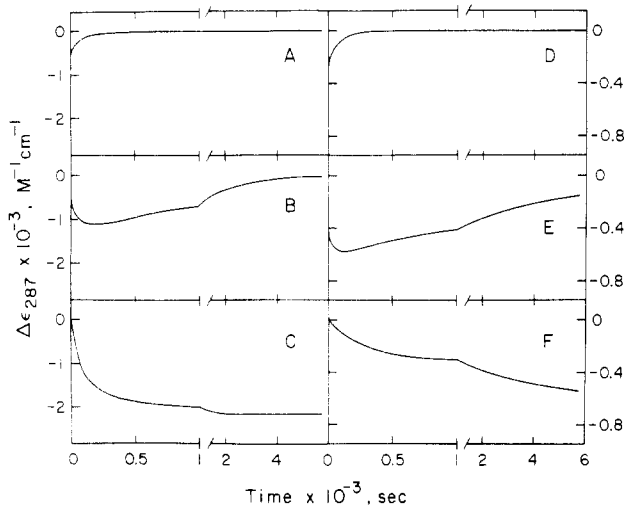

FIGURE 6: Comparison between unmodified RNase A (left panels) and CL(7–41)-RNase A (right panels) showing the existence of a nativelike intermediate trapped at 0 °C. The protein (7.3×10^{-3} M) in 6 M Gdn·HCl and 50 mM Gly·HCl (pH 2.0) was incubated for 3–4 h at 0 °C. Prefolding was performed with a 20-fold dilution of the unfolded protein in 0.8 M $(NH_4)_2SO_4$ and 50 mM sodium cacodylate (pH 5.8) at 0 °C for 15 s, and the protein concentration during the prefolding was 3.65×10^{-4} M in 0.3 M Gdn·HCl. The assay was carried out by diluting the prefolding solution 10-fold with 50 mM sodium cacodylate (pH 5.8) at 10 °C containing 0.3 M Gdn·HCl (A and D), 3 M Gdn·HCl (B), or 4.8 M Gdn·HCl (E) for refolding or 4 M Gdn·HCl (C) or 5.8 M Gdn·HCl (F) for further unfolding. Final protein concentration was 3.65×10^{-5} M.

and is dominated by the rate constant for the major slow-refolding phase, $U_S^{II} \rightarrow N$ (Hagerman & Baldwin, 1976). Above 4 M Gdn·HCl, the relaxation times differ for the two proteins. Under these conditions, the major kinetic phase in folding/unfolding is dominated by the rate constant for fast-phase unfolding, $N \rightarrow U_F$. Thus, the $U_S^{II} \rightarrow N$ rate appears to be the same for both proteins, while the rate for $N \rightarrow U_F$ differs considerably between the two cases.

The reversibility of Gdn·HCl denaturation was examined by column chromatography as described under Experimental Procedures. The results showed that the refolded unmodified RNase A or CL(7–41)-RNase A from the kinetic experiments coeluted from the ion-exchange column as a single peak with unmodified RNase A or CL(7–41)-RNase A, respectively, in the native conformation. No irreversibly denatured protein was detected in the chromatograms. Thus, we concluded that Gdn·HCl-denatured unmodified RNase A or CL(7–41)-RNase A refolds to the native conformation in the kinetic experiments.

Detection of a Nativelike Intermediate. Cook et al. (1979) found that the protein of the major slow phase of unmodified RNase A folds through a nativelike intermediate under strongly folding conditions. We examined the formation of a similar nativelike intermediate in the major slow phase of CL(7–41)-RNase A. Figure 6 shows the kinetic traces at pH 5.8 and 10 °C after a 15-s prefolding pulse under strongly folding conditions for unmodified RNase A (Figure 6A–C) and for CL(7–41)-RNase A (Figure 6D–F). During the prefolding pulse, the fast-folding species of unmodified RNase A are completely folded to native RNase A, and the major slow-folding species (U_S^{II}) are predominantly converted into a nativelike intermediate, but the minor slow-folding species (U_S^{I}) have yet to refold appreciably (Figure 7). As shown in Figure 7A, further incubation in 0.3 M Gdn·HCl at pH 5.8 and 10 °C allows the folding of the nativelike intermediate and the minor slow-folding species to the native conformation so that the absorbance at 287 nm increases continuously (Figure 6A). In 3 M Gdn·HCl at pH 5.8 and 10 °C, the nativelike intermediate is no longer stable, although native RNase A exists stably (Schmid, 1983). Thus, as shown in Figure 7B, during the further incubation in 3 M Gdn·HCl at pH 5.8 and 10 °C, the nativelike intermediate first unfolds to the major slow-folding species, leading to a decrease in absorbance in the early stages of the incubation. Then the major and minor slow-folding species refold to the native conformation, giving a subsequent absorbance increase at 287 nm (Figure 6B). In 4 M Gdn·HCl at pH 5.8 and 10 °C, as shown in Figure 7C, neither the nativelike intermediate nor the native protein is stable; so, further incubation under these conditions unfolds the nativelike intermediate and native

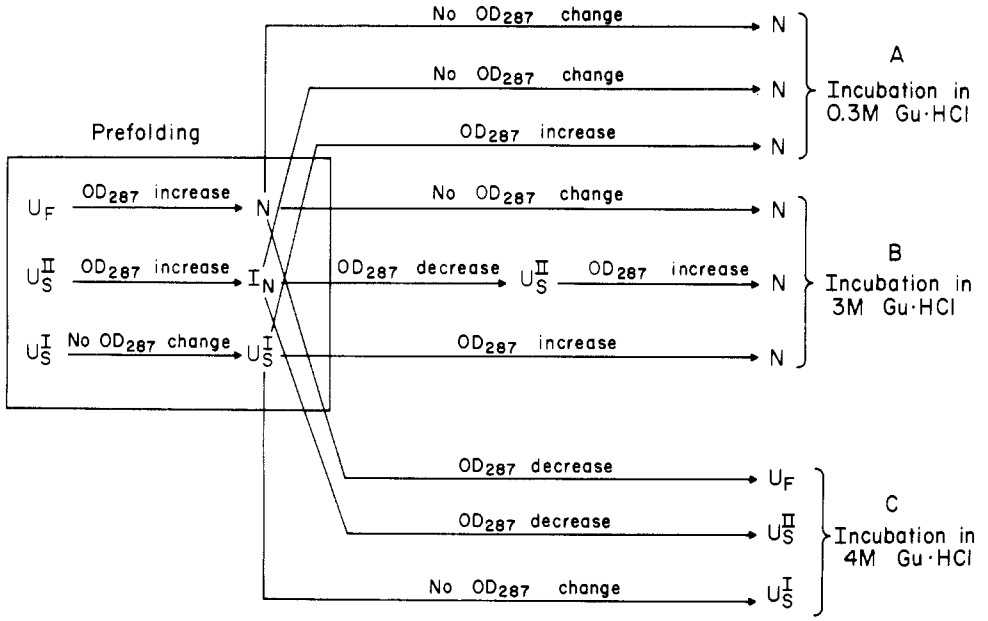

FIGURE 7: Schematic diagram of the double-jump experiments showing the change of the species and absorbance of unmodified RNase A. (A), (B), and (C) correspond to panels A, B, and C, respectively of Figure 6. A similar diagram, which differs in the Gdn·HCl concentrations required in the final assay conditions, may be constructed for CL(7–41)-RNase A. The detailed experimental conditions are given in Figure 6.

RNase A, giving a continuous decrease in absorbance at 287 nm (Figure 6C).

These same phenomena were observed for CL(7–41)-RNase A (Figure 6D–F) except that *higher* Gdn·HCl concentrations were required to denature the nativelike intermediate and the native conformation, compared to those for unmodified RNase A. Thus, we conclude that the major slow-folding species in Gdn·HCl-denatured CL(7–41)-RNase A also folds through a nativelike intermediate under strongly folding conditions and that the nativelike intermediate in CL(7–41)-RNase A is more stable against unfolding by Gdn·HCl than that in unmodified RNase A. This implies that a part or all of the 41 N-terminal residues are folded in the nativelike intermediate and that the cross-link destabilizes the unfolded state rather than the nativelike intermediate; as a result, the latter is stabilized against unfolding.

Discussion

It is very important to be able to provide a conformational analysis of the species that exists at the transition state in a kinetic protein folding/unfolding experiment. However, this is difficult to carry out because the transition-state species has a high free energy and never accumulates to a significant extent. Nevertheless, in the following discussion, we are able to draw conclusions about the conformation of the species in the transition state in the folding/unfolding pathways for RNase A.

Folding Pathways. Although the extrinsic cross-link used in this study forms a closed loop from Lys^7 to Lys^{41}, in the following analysis we consider that the entire 41 N-terminal residues have the same degree of folding as the loop residues. This is reasonable because it is unlikely that residues from Thr^3 to Ala^6 would be in the native α-helix conformation if the residues from Lys^7 to Met^{13} were not, considering the cooperativity of the α-helix.

The native conformations are stable for both unmodified RNase A and CL(7–41)-RNase A at low concentrations of Gdn·HCl ($\leq$2 M) at pH 5.8. Thus, we studied the folding pathways under such folding conditions (Figures 2, 3, and 5). We observed no effect of the extrinsic cross-link on the folding rate constants in any of the fast-, major slow-, and minor slow-folding pathways. As shown in Figure 8A with a schematic conformational free energy diagram for the folding pathway, this means that the extrinsic cross-link destabilizes the transition state as well as the species that exist before the rate-limiting step (probably by decreasing the chain entropy of the 41 N-terminal residues). Thus, we conclude that the 41 N-terminal residues are unfolded in the transition state to the same extent as in the kinetically trapped intermediate (I in Figure 8A) and fold to a nativelike structure *after* the rate-limiting step. Also, the 41 N-terminal residues make no conformational contribution to enhance the refolding kinetics.

However, this does not mean that the 41 N-terminal residues do not participate in the kinetic refolding. In the kinetic refolding of RNase S, S peptide associates with S protein before the rate-limiting steps and enhances the refolding rates (Labhardt & Baldwin, 1979a,b; Labhardt et al., 1983). Thus, the N-terminal residues could be contributing to the kinetic refolding by conformationally independent associations(s) or interactions with the rest of the protein which have yet to be characterized.

Unfolding Pathway. The denatured conformations are stable for both unmodified RNase A and CL(7–41)-RNase A at high concentrations of Gdn·HCl ($\geq$6 M) at pH 5.8. Thus, we studied the unfolding pathways under such unfolding conditions, observing a significant difference in the unfolding

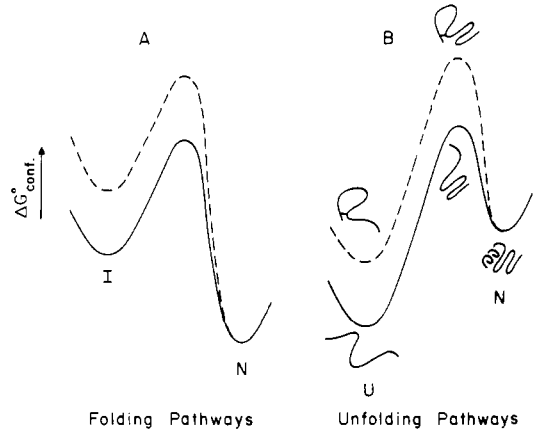

FIGURE 8: Schematic conformational free energy ($\Delta G^\circ_{conf.}$) for the folding (A) and unfolding (B) pathways of unmodified RNase A (solid lines) and CL(7–41)-RNase A (dashed lines). In (A), "I" is the kinetically trapped intermediates of unmodified RNase A and CL(7–41)-RNase A in the folding pathways. "N" represents the native state of both proteins. The fast-, major slow-, and minor slow-folding pathways are not distinguished in the figure. The conformational activation free energy for each of the three pathways is the same for the corresponding pathways in both unmodified RNase A and CL(7–41)-RNase A, so that the free energy difference in the kinetically trapped intermediates still persists at the transition states. The conformational free energy of the native state is the same for both proteins (Lin et al., 1984). (B) "U" is the disordered conformations of unmodified RNase A and CL(7–41)-RNase A in the unfolding pathway. "N" represents the native state for both proteins. The conformations of the proteins are shown schematically, emphasizing that the 41 N-terminal residues are unfolded in the transition state of the unfolding pathway.

kinetics between unmodified RNase A and CL(7–41)-RNase A (Figures 4 and 5). Since the unfolding rate constant is much larger than the folding rate constant under unfolding conditions, the relaxation time of the kinetic unfolding can be approximated as the inverse of the unfolding rate constant. Assuming the same unfolding process for unmodified RNase A and CL(7–41)-RNase A, the difference in the unfolding rate constants between unmodified RNase A and CL(7–41)-RNase A (i.e., 9.0 and 580 s, respectively, in Figure 4) corresponds to a difference of 2.5 kcal/mol in the activation free energy. The conformational free energy diagram for the unfolding pathways of unmodified RNase A and CL(7–41)-RNase A is shown in Figure 8B. This diagram includes the information that the extrinsic cross-link destabilizes the denatured state, probably by reducing its chain entropy, but not the native state (Lin et al., 1984). Since the equilibrium constant between the native and denatured states is the ratio of the unfolding and folding rate constants, under the unfolding conditions of Figure 8B, the effect of the cross-link on the equilibrium constant (viz., 3.0 kcal/mol in standard free energy) arises only from the change in the unfolding rate constant (cf. 3.0 and 2.5 kcal/mol), with no effect on the folding rate constant, as observed under the folding conditions of Figure 8A. It is also reasonable to conclude that the cross-link destabilizes the transition state on the unfolding pathway, probably by decreasing the chain entropy of the protein in the denatured state. This interpretation means that the unfolding process is *sequential* in the sense that the 41 N-terminal residues must be unfolded in the transition state as drawn schematically in Figure 8B.

Presumably, if the cross-link were introduced instead between two *other* residues that are in the segment(s) involved in the rate-limiting step(s), it could increase the refolding rate constants and possibly the concentration of fast-folding species (Scheraga et al., 1984).

Conclusion

On the basis of kinetic measurements for the folding/unfolding of RNase A with/without an extrinsic cross-link, we conclude that RNase A unfolds *sequentially* and that the 41 N-terminal residues must be unfolded in the transition state of the unfolding pathway. Also, the 41 N-terminal residues make no conformational contribution in the folding pathway.

Added in Proof

P. C. Weber, D. O. Ohlendorf, B. Finzel, S. Sheriff, and F. R. Salemme (private communication) have determined the crystal structure of this cross-linked protein to 2-Å resolution, refined by restrained least-squares methods to an R factor of 0.19. The structure is essentially that of native RNase, with a root mean square deviation on all backbone atoms of 0.52 Å and over all side-chain atoms of 1.34 Å. The *small* differences observed between these two structures are comparable to coordinate differences typically observed for identical proteins crystallized in different crystal forms.

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References

Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.
Borkakoti, N., Moss, D. S., & Palmer, R. A. (1982) *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem. B38*, 2210.
Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157.
Denton, J. B., Konishi, Y., & Scheraga, H. A. (1982) *Biochemistry 21*, 5155.
Garel, J.-R., Nall, B. T., & Baldwin, R. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1853.
Ginsburg, A., & Carroll, W. R. (1965) *Biochemistry 4*, 2159.
Goto, Y., & Hamaguchi, K. (1982a) *J. Mol. Biol. 156*, 891.
Goto, Y., & Hamaguchi, K. (1982b) *J. Mol. Biol. 156*, 911.
Hagerman, P. J., & Baldwin, R. L. (1976) *Biochemistry 15*, 1462.
Johnson, R. E., Adams, P., & Rupley, J. A. (1978) *Biochemistry 17*, 1479.
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.
Lin, L.-N., & Brandts, J. F. (1983) *Biochemistry 22*, 564.
Lin, S. H., Konishi, Y., Denton, M. E., & Scheraga, H. A. (1984) *Biochemistry 23*, 5504.
Lynn, R. M., Konishi, Y., & Scheraga, H. A. (1984) *Biochemistry 23*, 2470.
Nall, B. T., & Landers, T. A. (1981) *Biochemistry 20*, 5403.
Nozaki, Y. (1972) *Methods Enzymol. 26*, 43.
Scheraga, H. A., Konishi, Y., & Ooi, T. (1984) *Adv. Biophys. 18*, 21.
Schmid, F. X. (1983) *Biochemistry 22*, 4690.
Segawa, S. (1984) *Biopolymers 23*, 2489.
Takahashi, S., & Ooi, T. (1973) *Bull. Inst. Chem. Res., Kyoto Univ. 51*, 329.
von Hippel, P. H., & Wong, K.-Y. (1965) *J. Biol. Chem. 240*, 3909.

Contact Site of Histones 2A and 2B in Chromatin and in Solution†

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ABSTRACT: Irradiation of isolated nuclei or of a complex of histones 2A (H2A) and 2B (H2B) with ultraviolet light produces a covalent cross-link between H2A and H2B. Sequence analysis of the peptides isolated from the H2A–H2B dimer formed in solution and in nuclei demonstrated that both dimers are produced through the covalent linkage of Tyr-40 of H2B and Pro-26 of H2A. Tyrosyl residues proximal to Tyr-40 did not produce a cross-link with H2A, thereby indicating that strict conformational parameters are required for production of the H2A–H2B cross-link. We conclude that the precise juxtaposition of Tyr-40 of H2B and Pro-26 of H2A in this region of the H2A/H2B contact site is not altered upon interaction of these histones with H3 and H4 (tetramer), DNA, or other chromosomal components during nucleosome assembly.

Chromatin is composed of DNA associated with a heterogeneous protein component, of which the histones are the most prevalent class. The substructure of chromatin includes the nucleosome, a histone octamer wrapped in superhelical DNA associated with histone 1. A limited nuclease digest of chromatin produces a core particle of 146 base pairs of DNA surrounding core histones 2A, 2B, 3, and 4 [for reviews, see Kornberg (1977) and McGhee & Felsenfeld (1980)]. Histones 3 and 4 can form a tetramer that interacts with short strands

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