Circular Dichroism Evidence for the Presence of Burst-Phase Intermediates on the Conformational Folding Pathway of Ribonuclease A†

Walid A. Houry, David M. Rothwarf, and Harold A. Scheraga*

Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301

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ABSTRACT: Refolding of the very-fast-folding unfolded species (U_{vf}) of disulfide-intact bovine pancreatic ribonuclease A has been monitored by circular dichroism (CD) at 222 and 275 nm at 0.9 or 2.6 M guanidine hydrochloride, pH 7.0, and 5 °C. The refolding of U_{vf} represents a purely conformational folding process which is not complicated by *cis*−*trans* proline isomerization. The data indicate that there are at least two intermediates on the refolding pathway of U_{vf} and that both intermediates form in the burst phase when the refolding is monitored by CD. At the initiation of folding, U_{vf} is converted to a largely unfolded intermediate, termed I_U, which then undergoes a hydrophobic collapse to form the molten-globule-like intermediate I_Φ. The CD values obtained for I_U and I_Φ indicate that I_U has no significant secondary structure and presumably differs from U_{vf} by a local structural rearrangement, while I_Φ has a substantial population of secondary and tertiary structures, about 40%−50% of that of native.

The protein folding problem involves the determination of the three-dimensional structure of the protein solely on the basis of its amino acid sequence. In order to solve this problem, many attempts have been made to try to understand the principles that govern the interactions among the amino acid residues which result in the formation of secondary and tertiary structures in proteins. These attempts have been both theoretical (Scheraga, 1992; Thomas, 1992; Bryngelson et al., 1995; Dill et al., 1995; Hao & Scheraga, 1995; Karplus & Sali, 1995) and experimental (Montelione & Scheraga, 1989; Kim & Baldwin, 1990; Matthews, 1993; Fersht, 1993; Baldwin, 1995; Creighton et al., 1996). From the experimental point of view, attempts are usually made to draw a picture of the folding pathway of the protein as it proceeds from its unfolded state, which is thought to be a statistical coil, to its well-defined native state. Along this folding pathway, intermediates are formed and depleted, transition state barriers are overcome, and unproductive folding events are avoided or minimized.

These folding pathways are typically highly complicated in the kinetic sense. Nevertheless, attempts are made to investigate the nature of the events that occur early in the folding reaction and to determine the structures of the intermediates that are formed along the folding pathway. The difficulty in these studies lies in the fact that the unfolded state of the protein is usually a mixture of unfolded species and that the important intermediates are usually formed within the dead time of the kinetic instrument. Nonetheless, progress has been made to overcome these difficulties by combining different experimental techniques in studying some typical proteins.

Disulfide-intact bovine pancreatic ribonuclease A (RNase A)[1] is an example of such a protein. Its folding pathway has been studied extensively. The unfolded state of RNase A is a mixture of unfolded species. This heterogeneity in the unfolded state of the protein arises primarily from the *cis*−*trans* isomerization of the X-Pro peptide bonds in the unfolded state (Brandts et al., 1975; Schmid, 1986; Schultz et al., 1992; Nall, 1994). In RNase A, there are four X-Pro peptide bonds. When the protein is in its native state, two of these peptide bonds (X-Pro 93 and 114) adopt the *cis* conformation; the other two peptide bonds (X-Pro 42 and 117) adopt the *trans* conformation (Wlodawer et al., 1988). When the protein unfolds, each of these X-Pro peptide bonds isomerizes to adopt the *cis* as well as the *trans* conformation. On the basis of studies of model peptides (Dyson et al., 1988), the equilibrium distribution of these peptide bonds is estimated to be about 10%−20% in the *cis* conformation and about 80%−90% in the *trans* conformation.

In our previous studies (Houry et al., 1994; Dodge & Scheraga, 1996), we have proposed that the unfolded state of RNase A consists of at least eight unfolded species. Each of these species has a different combination of conformations at the X-Pro peptide bonds. We have shown that only one of these unfolded species has the native conformation at all the X-Pro peptide bonds. We call this species U_{vf} (a very-fast-folding species). The folding of U_{vf} is not complicated by the isomerization around the X-Pro peptide bonds. Hence, its folding is a purely conformational process which was elucidated previously (Houry et al., 1995). At low pH and low denaturant concentrations, it was shown experimentally that the major initial event in the folding process of U_{vf} is a hydrophobic collapse which results in the early formation

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* Author to whom correspondence should be addressed.

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[1] Abbreviations: RNase A, disulfide-intact bovine pancreatic ribonuclease A; CAM-RNase A, fully reduced iodoacetamide-blocked bovine pancreatic ribonuclease A; GdnHCl, guanidine hydrochloride; CD, circular dichroism with values expressed in terms of mean residue ellipticity; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid. U_{vf}, the very-fast folding species of RNase A; U_{eq}, the equilibrium unfolded state of the protein consisting of all the unfolded species.

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of an intermediate. This intermediate, labeled I_Φ, has properties similar to those of molten globules (Houry et al., 1995). I_Φ is highly populated at low pH and low denaturant concentrations, and its presence explains the unexpected kinetics observed under these conditions. At higher pH and higher denaturant concentrations, the population of the intermediate decreases and the folding reaction approximates a two-state process. The folding process of U_{vf} proposed in our previous study (Houry et al., 1995) can be written as follows:

$$U_{vf} \overset{K}{\rightleftharpoons} I_\Phi \overset{k}{\rightarrow} N \qquad (1)$$

where N is the native species. When the folding of U_{vf} is initiated, there is a rapid pre-equilibrium to form the intermediate. This is described by the equilibrium constant $K = [I_\Phi]/[U_{vf}]$, and k is the rate constant for the formation of N from I_Φ.

The native structure of RNase A consists of three helices (Wlodawer et al., 1982) which span residues 3−13 (helix I), 24−34 (helix II), and 50−60 (helix III). The rest of the protein consists of β-strands, turns, β-bulges, and other structures. In addition, the protein contains six tyrosines; three of these tyrosines are buried in the native state of the protein (Scheraga, 1967; Wlodawer et al., 1988; Santoro et al., 1993). Previously, we have followed the folding of U_{vf} by absorbance (Houry et al., 1994, 1995; Dodge & Scheraga, 1996) and by fluorescence (Houry et al., 1994) which monitor the change in the environment of the tyrosine residues. In the present study, the folding of U_{vf} was monitored by circular dichroism (CD).

Circular dichroism is a spectroscopic method that allows for the monitoring of various secondary structures and of various tertiary contacts in the protein. CD arises from the asymmetry of a chromophore. CD spectra in the far-UV region (222 nm) are attributed mainly to secondary-structure content (Johnson, 1990; Woody, 1995). Spectra in the near-UV region (275 nm) probe mainly the environment of aromatic groups and disulfides in the protein (Kahn, 1979) and, hence, provide information about the tertiary contacts (structure) present in the protein.

When the folding of U_{vf} was monitored by absorbance at 287 nm, the refolding amplitude remained unchanged under the wide range of pH and GdnHCl concentrations employed, and no burst phase could be observed by absorbance for the formation of an intermediate (Houry et al., 1995). This indicated that the formation of the intermediate, I_Φ, does not result in significant burial of the tyrosine side chains. Hence, we concluded that the absorbance properties of the intermediate are similar to those of the unfolded state within the experimental error of the instrument used. In the current study, the folding of U_{vf} was monitored by CD under two different conditions: one under which I_Φ is expected to be highly populated (favorable conditions) and another under which I_Φ is expected to be present only at negligible concentrations (unfavorable conditions). The kinetics and amplitudes as observed by CD under these two different conditions were compared. A burst phase could be observed by CD under conditions in which the intermediate is expected to be highly populated, and a smaller burst was also observed under the unfavorable condition. The implications of these observations on the folding pathway are discussed.

MATERIALS AND METHODS

Reagents. Glycine (Gly), iodoacetamide, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)-propanesulfonic acid (MOPS), and tris(hydroxymethyl)-aminomethane (Tris) were purchased from Sigma Chemical Company. Acetic acid, HCl, NaCl, Na_2EDTA, and NaOH were obtained from Fisher Scientific Company. Guanidine hydrochloride (GdnHCl), ultra pure, was obtained from ICN Biochemicals, and guanidine thiocyanate (GdnSCN) was purchased from J. T. Baker.

Protein Purification. RNase A, types I-A and II-A, was purchased from Sigma and was purified as described previously (Rothwarf & Scheraga, 1993). The purity of the protein was checked by cation-exchange chromatography using a Hydropore-5-SCX column (Rainin) on a Spectra Physics 8700 HPLC system. The protein was found to be >99% pure.

Iodoacetamide-blocked RNase A (CAM-RNase A) was prepared as follows. Reduced RNase A was prepared as described previously by Rothwarf and Scheraga (1993). The reduced protein, at a concentration of about 4 mg/mL, was then blocked with 170 mM iodoacetamide in 100 mM Tris, 2 mM Na_2EDTA, 1 M GdnSCN, pH 8.0, for 30 min in the dark and immediately desalted into 100 mM Tris, 2 mM Na_2-EDTA, pH 8.0, using a disposable PD10 column (Pharmacia). It was desalted again into 100 mM acetic acid on an HR 16/50 column (Pharmacia) packed with G-25 superfine resin (Pharmacia). An LKB 2150 pump and an Isco UA-5 detector with a type 9 optical unit and 280 nm filters constituted the solvent delivery and detection system. The material was then lyophilized. It was purified by cation-exchange chromatography on a Rainin Hydropore SCX 21 mm × 10 cm column in 25 mM HEPES, 1 mM Na_2EDTA, pH 7.0, using a linear gradient from 0 to 200 mM NaCl in 80 min at a flow rate of 8 mL/min. An LKB 2249 gradient system with an Isco UA-5 detector having a Type 9 optical unit and 280 nm filters was used. The purified material was desalted into 100 mM acetic acid using the same HR 16/50 column described above and then lyophilized.

GdnHCl Transition Curves. To measure the change in the CD signal of the disulfide-intact ribonuclease A (RNase A) as a function of GdnHCl concentration (Figure 1) at 5 °C, the following procedure was followed. A stock solution of RNase A was made by dissolving the protein in distilled deionized water to a concentration of 4.83 mg/mL for CD measurements at 222 nm and to a concentration of 48.25 mg/mL for CD measurements at 275 nm. The pH of these stock solutions was 4.9 at room temperature. The concentration of the protein was determined by dilution using an absorbance extinction coefficient of 9800 M^{-1} cm^{-1} at 277.5 nm (Sela & Anfinsen, 1957) on a modified Cary 14 spectrophotometer.

The GdnHCl buffers contained 45 mM MOPS, and their pH was adjusted to 6.8 (±0.1) at room temperature with concentrated HCl and NaOH. The concentration of GdnHCl was determined by refractive index at 25 °C using a Bausch & Lomb refractometer (Nozaki, 1972). The uncertainty in the GdnHCl concentration was estimated to be ±0.05 M. All solutions were filtered through a 0.2 μ filter (Gelman).

To carry out the CD measurements at 222 nm, 35 μL of the 4.83 mg/mL RNase A stock solution was dissolved in

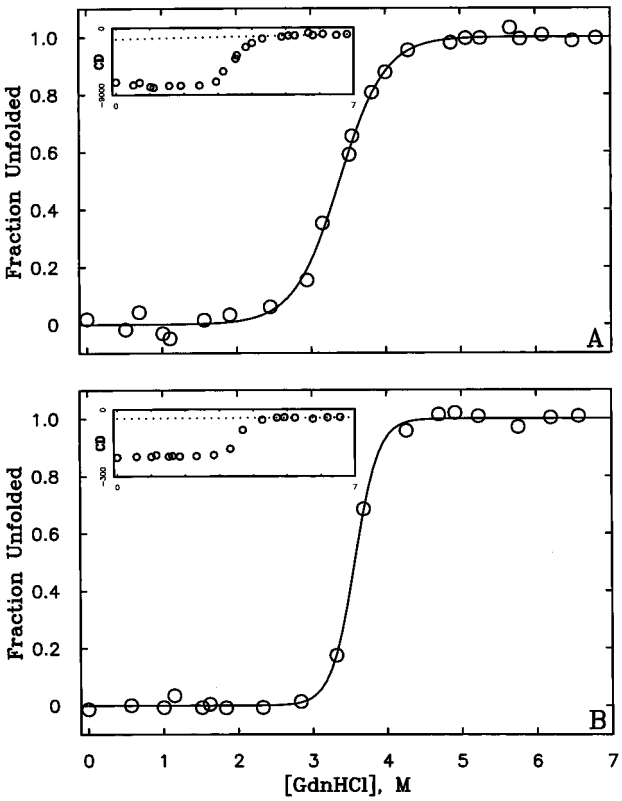

FIGURE 1: GdnHCl-dependence curves at pH 7.0 and 5 °C as monitored by CD at (A) 222 nm and at (B) 275 nm, converted to fraction unfolded. The solid lines are the fit to the raw experimental data based on the procedure of Santoro and Bolen (1988). The insets give the actual CD values [in (deg·cm^2/dmol)] as a function of GdnHCl concentration (in M). The dotted lines in the insets are the linear fits to the post-transition regions. The midpoint of the transitions are (A) 3.4 (±0.2) M and (B) 3.6 (±0.2) M.

700 μL of the GdnHCl buffers. This resulted in solutions of 0.23 mg of RNase A/mL, 43 mM MOPS, and a measured pH of 7.0 (±0.1) at 5 °C at the resulting GdnHCl concentration. These solutions were sonicated for about 25 min and then placed in the cold room (temperature of 4−5 °C) until their CD was measured (after at least 12 h).

The CD measurements at 275 nm were carried out by dissolving 40 μL of the 48.25 mg/mL RNase A stock solution in 700 μL of the GdnHCl buffers. This resulted in solutions of 2.6 mg of RNase A/mL, 43 mM MOPS, and a measured pH of 7.0 (±0.1) at 5 °C at the resulting GdnHCl concentration. The solutions were treated as described above.

The reference buffers were prepared and treated in the same way as the protein-containing samples, except that 35 or 40 μL of distilled deionized water was added to the GdnHCl buffers, instead of the protein stock solution.

The CD measurements were carried out on a modified Cary model 14 spectrophotometer (Adler & Scheraga, 1988) using a sensitivity of 0.1 and a slit width of 3 mm connected to a SUN workstation. All measurements were carried out at 5 °C. The temperature was maintained using a Poly-Science model 9110 circulating bath. The temperature in the cell was measured using a calibrated thermistor (Fisher). A 2 mm pathlength cuvette (NSG Precision Cells, Inc.) was used to measure the CD of the samples. The sample cell holder was flushed with N_2 to prevent condensation. 700 μL of reference buffer was first placed in the cuvette and allowed to equilibrate at 5 °C for 20 min. A time scan for 100 s was obtained at a scan rate of 1 measurement/second. Then without moving the cuvette, the cuvette was cleaned and 700 μL of the protein sample was placed in the cuvette and allowed to equilibrate for 20 min. A time scan was then obtained as for the reference buffer. The average CD of the buffer was subtracted from the average CD of the protein sample to obtain the final CD, in degrees, of the protein solutions at each GdnHCl concentration. This was then converted to mean residue ellipticity using the equation

$$[\theta]\ (\mathrm{deg\cdot cm^2/dmol}) = [\theta\ (\mathrm{deg})]/\{[\text{protein concentration } (\mathrm{dmol/cm^3})]\cdot[\text{cuvette pathlength (cm)}]\cdot[\text{no. of aa in protein}]\} \quad (2)$$

Kinetic Measurements. The double-jump CD kinetic measurements were carried out on an SFM-3 Bio-Logic stopped-flow instrument that contained three syringe drives. The instrument was connected to an Aviv CD62 spectropolarimeter which allowed for the rapid monitoring of the change in the CD signal as the protein proceeded from its unfolded state to its folded state. A 450 W Xe lamp was used. The cell pathlength was 2 mm, and the dead time of the instrument was estimated to be 5 ms. At 222 nm, the slit width was set at 1.573 mm, while at 275 nm, the slit width was set at 0.691 mm. Data were collected on a PC using the Bio-Kine software version 3.23 from Biologic. The temperature of the syringes, mixers, flow cell, and the connecting lines was maintained constant with a Lauda RCS-6 circulating bath. Buffers were degassed by aspiration prior to use.

All kinetic experiments were carried out in a double-jump mode. The procedure for these experiments is similar to that employed previously (Houry et al., 1995). Double-jump experiments consist of two mixing events (jumps). In the first jump, the native protein is unfolded, and then, in the second jump and after a set delay time, the protein is refolded. The refolding of U_{vf} was monitored by CD at 222 and at 275 nm. The temperature in all of the experiments was maintained at 5 °C. The final GdnHCl concentrations are known with an uncertainty of ±0.1 M.

At 222 nm, two different conditions were employed in the kinetic experiments. In these experiments, the protein was first unfolded at 4.2 M GdnHCl and pH 2.0 by diluting 1.5 M GdnHCl, 50 mM MES, pH 6.0, containing 4.44 mg of RNase A/mL into 5.28 M GdnHCl, 40 mM Gly, pH 1.53, in a ratio of 1:2.5 for about 1 s. This time is long enough to form >99% U_{vf}; however, it is short enough to prevent X-Pro peptide bond isomerization in the unfolded state and, hence, prevents the formation of the other unfolded species. After 1 s, U_{vf} was then refolded at 0.9 M GdnHCl and pH 7.0 [where I_Φ is expected to be highly populated (Houry et al., 1995)], or at 2.6 M and pH 7.0 [where no significant population of I_Φ is expected to be present (Houry et al., 1995)]. This was achieved by diluting the unfolded protein with 0 M GdnHCl, 50 mM MOPS, pH 7.1, or 2.16 M GdnHCl, 50 mM MOPS, pH 7.0. The final protein concentration was 0.27 mg/mL.

At 275 nm, one condition was employed. The protein was first unfolded at 4.2 M GdnHCl and pH 2.0 for 1 s to form >99% U_{vf} by diluting 1.5 M GdnHCl, 50 mM MES, pH 5.7 containing 61.4 mg of RNase A/mL with 5.28 M GdnHCl, 40 mM Gly, pH 0.94. Then, after 1 s, the protein was refolded at 0.9 M GdnHCl and pH 7.0 by diluting the

unfolded protein with 0 M GdnHCl, 50 mM MOPS, pH 7.2. The final protein concentration was 3.8 mg/mL.

All of the kinetic experiments were repeated many times, typically 40−80 times, in order to achieve a reasonable signal to noise ratio.

The mixing ratios in the SFM-3 Biologic stopped-flow instrument are determined by the speed with which the syringes containing the different buffers are driven. Each syringe is driven by a separate electronic stepper motor. This results in about a 10% uncertainty in the mixing ratios. We have, therefore, determined the final protein concentration that was present in the flow cell at the end of the second jump, by comparing the CD value obtained for the native state from the kinetic refolding curves with the CD value obtained from the steady-state measurements (described below) for the native protein.

In order to determine whether there is a difference in the CD at 222 nm between U_{vf} and the equilibrium unfolded state (U_{eq}) which consists of all the unfolded species (see Discussion), the following double-jump experiment was carried out by manual mixing. The native protein (4.44 mg/mL) in 1.5 M GdnHCl, 50 mM MES, pH 6.0, was unfolded at 4.2 M GdnHCl and pH 2.0 as above. Then the unfolded protein was jumped into 5.9 M GdnHCl and pH 7.0 by dilution with 6.36 M GdnHCl, 50 mM MOPS, pH 7.1. The CD signal was then observed at 222 nm using a 2 mm pathlength cell. Each mixing event took about 10 s to carry out. After 10 s of unfolding at 4.2 M GdnHCl and pH 2.0, the population of U_{vf} is about 90% [from data of Houry et al. (1994)]. The final protein concentration was 0.27 mg/mL. This procedure was repeated four times. No change in the CD signal could be observed when the solution conditions were changed from 4.2 M GdnHCl, pH 2.0, to 5.9 M GdnHCl, pH 7.0.

Steady-State CD Measurements. In order to compare the results from the kinetic experiments with those from equilibrium measurements properly, the equilibrium CD measurements were carried out with the same buffers in the same ratios as those used for the kinetic experiments.

These experiments were carried out as follows. The lyophilized protein, either the disulfide-intact ribonuclease A (RNase A) or the iodoacetamide-blocked ribonuclease A (CAM-RNase A), was dissolved in 100 mM acetic acid to a concentration of about 1 mg/mL. The concentration was measured on a modified Cary 14 spectrophotometer at 275 nm using an extinction coefficient of 9300 M^{-1} cm^{-1} for RNase A (Konishi & Scheraga, 1980) and a value of 8160 M^{-1} cm^{-1} for CAM-RNase (Rothwarf & Scheraga, 1993). The value of 8160 M^{-1} cm^{-1} had been determined for reduced and unblocked protein. It is unlikely that the blocking groups would significantly change the absorbance properties of the reduced protein.

Appropriate aliquots of the protein solutions were then placed into tubes and lyophilized. The dry lyophilized protein was then dissolved in appropriate volumes of the CD buffers which were made by mixing the relevant buffers used in the kinetic experiments to give the same final composition as that obtained in the flow cell after the second jump in the double-jump experiments (except for the 4.2 M GdnHCl solution which was made by mixing the 5.9 M GdnHCl solution with the 0.9 M GdnHCl solution). The final protein concentration was 21.2 μM for RNase A (0.29 mg/mL) and CAM-RNase A for CD measurements at 222 nm and 4.0 mg/mL for CD measurements at 275 nm.

Data Fitting. The decay curves from the double-jump kinetic experiments were fit to single exponential curves using the program PLOT from New Unit (Ithaca, NY). The GdnHCl transition curves were fit according to the procedure described by Santoro and Bolen (1988). A simplex algorithm (Caceci & Cacheris, 1984) was used to minimize $\chi^2 = \sum[y_{experimental} - y_{calculated}]^2$. All errors in the data are given as standard deviations.

RESULTS

GdnHCl Transition. The GdnHCl transition curves as monitored by CD at 222 and 275 nm are shown in Figure 1A and B, respectively. The solid curves in the figures are the fit to the experimental points based on the equation given by Santoro and Bolen (1988). Several important parameters can be obtained from fitting the GdnHCl transition curves. However, only the width of the transition region and the slope of the post-transition region are important for the current study.

The midpoints of the transitions as obtained by CD(222 nm) and CD(275 nm) are 3.4 (±0.2) M and 3.6 (±0.2) M, respectively. The two values are similar within experimental error. In both curves, the transition region extends from about 2.7 to 4.5 M GdnHCl. The kinetic experiments were carried out at 0.9 M, which lies within the pre-transition region, and at 2.6 M GdnHCl, which lies at the edge of the transition. One observation, that will be important later in the discussion, is that the slope of the linear fit to the post-transition region at 222 and 275 nm is very small (the dotted lines in the insets of Figure 1A and B). The slope of the line at 222 nm is 17 (deg·cm^2)/(dmol·M) (Fig 1A), and the slope of the line at 275 nm is 0.44 (deg·cm^2)/(dmol·M) (Fig 1B).

Kinetic Measurements. Steady-state and GdnHCl-dependence CD measurements of RNase A at 5.9 M GdnHCl, pH 7.0, and 5 °C, give the CD value for the equilibrium unfolded state (U_{eq}) which consists of all the different unfolded species; these unfolded species have different *cis* and *trans* isomers at each of the four X-Pro peptide bonds. However, in the kinetic refolding experiments, the reference state whose CD value is needed in order to measure the magnitude of the burst phase properly is U_{vf}. In other words, a question arises as to whether U_{vf} and U_{eq} have different CD values. In order to answer this question, a manual mixing double-jump experiment was carried out as described in Materials and Methods. In this experiment, the folded protein was unfolded at 4.2 M GdnHCl and pH 2.0 to form U_{vf} and then kept unfolded with a change of solvent to 5.9 M GdnHCl and pH 7.0 while monitoring the change in CD at 222 nm. During this time, *cis*−*trans* X-Pro peptide bond isomerization takes place and the different unfolded species form from U_{vf}. In other words, U_{eq} forms from U_{vf} in the second jump. The relaxation time from U_{vf} to U_{eq} under the conditions used is expected to be on the order of 265 s (Houry et al., 1994) which is a long enough time to be observed by manual mixing. Since no change in the CD signal was observed, we can conclude that U_{vf} and U_{eq} have the same CD value at 222 nm, and, consequently, we expect them to have the same CD value at 275 nm (i.e., since they do not contain any significant secondary structure and since they have the

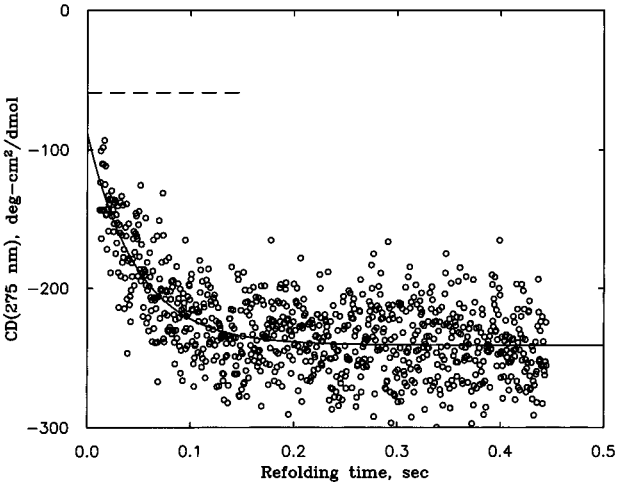

FIGURE 2: Refolding curve of U_{vf} at 0.9 M GdnHCl, pH 7.0, and 5 °C, as monitored by CD at 275 nm. The protein in 1.5 M GdnHCl, pH 5.7, was unfolded at 4.2 M GdnHCl and pH 2.0 for 1 s, resulting in the formation of >99% U_{vf}. U_{vf} was then refolded at 0.9 M GdnHCl, pH 7.0, and the refolding process was monitored by CD at 275 nm. The curve shown is the average of 80 repeats. The solid line is a single exponential fit to the experimental points. The dashed line shows the CD value of U_{vf} (see text).

same CD value at 222 nm, then they are unlikely to differ in their tertiary structure). Kiefhaber and Baldwin (1995) observed similar unfolding kinetics by CD at 222 and 275 nm. This further supports the conclusion that U_{vf} and U_{eq} have similar CD values at 222 and 275 nm, respectively.

In the kinetic experiments, all the refolding steps were carried out at pH 7.0 and 5 °C. Figure 2 shows the decay curve obtained for the refolding of U_{vf} at 0.9 M GdnHCl when monitored by CD at 275 nm. The curve is an average of 80 repeats. Figure 3A and B show the refolding curves of U_{vf} as monitored by CD at 222 nm at 2.6 and 0.9 M GdnHCl, respectively. The curve in Figure 3A is an average of 40 repeats, while that in Figure 3B is an average of 80 repeats. The dashed lines in Figures 2 and 3 represent the CD value of U_{vf} (or U_{eq}) at 5.9 M GdnHCl as obtained from the steady-state measurements (see below). When the refolding is carried out at 0.9 M GdnHCl, a large population of I_Φ is expected to be present, while at 2.6 M GdnHCl, I_Φ is not expected to be present to any significant extent (Houry et al., 1995). A single-exponential fit to the curves gives the refolding time constants (listed in Table 1) and the CD values at zero and infinite refolding times (listed in Table 2). The CD values at "zero" refolding time (line 1 in Table 2) when compared to the CD value of U_{vf} give the magnitude of the burst phase, while the CD values at infinite refolding time (line 2 in Table 2) give the CD value of the native (folded) protein.

Steady-State Measurements. Some of the data in Table 2 were obtained from steady-state measurements of RNase A and CAM-RNase A. These measurements were carried out using the same buffers as those used for the kinetic experiments mixed in the same ratio in order to avoid any effects on the CD signal arising from differences in solvent. The GdnHCl-dependence data were obtained using different composition of buffers from those used in the kinetic experiments. Therefore, in comparing kinetic measurements to equilibrium measurements it is more appropriate to use the values from the steady-state data than to use the values from the GdnHCl-dependence data. In the Discussion, we will compare the CD values from kinetic experiments only with those from steady-state measurements.

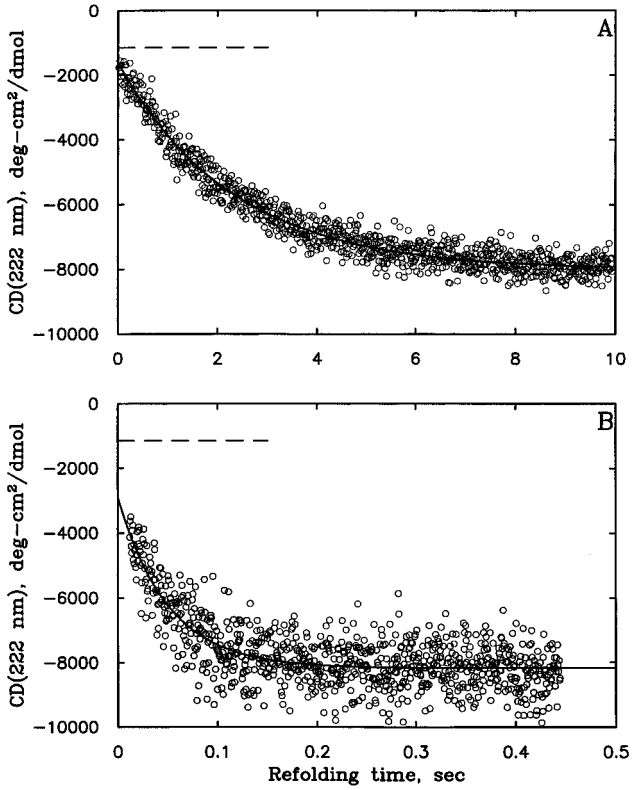

FIGURE 3: Refolding curve at U_{vf} at (A) 2.6 or (B) 0.9 M GdnHCl, pH 7.0, and 5 °C, as monitored by CD at 222 nm. The protein in 1.5 M GdnHCl, pH 6.0, was unfolded at 4.2 M GdnHCl and pH 2.0 for 1 s, resulting in the formation of >99% U_{vf}. U_{vf} was then refolded at (A) 2.6 or (B) 0.9 M GdnHCl, pH 7.0, and the refolding process was monitored by CD at 222 nm. The curve shown in A is the average of 40 repeats, and that shown in B is the average of 80 repeats. The solid lines are single exponential fits to the experimental points. The dashed lines show the CD value of U_{vf} (see text).

Table 1: Time Constants for the Refolding of U_{vf}, Measured with CD and Absorbance Spectroscopy[a]

	CD			absorbance	
wavelength (nm)	275	222	222	287	287
[GdnHCl] (M)[b]	0.9	0.9	2.6	0.9	2.6
τ(ms)[c]	47.8 (3.5)	50.3 (2.8)	2284 (37)	52.9	2172

[a] The folded protein in 1.5 M GdnHCl and pH 6.0 was unfolded at 4.2 M GdnHCl and pH 2.0 for 1 s, which is enough to obtain >99% U_{vf} without formation of the other unfolded species. U_{vf} was then refolded at 0.9 or 2.6 M GdnHCl and pH 7.0. All experiments were carried out at 5 °C. The numbers in parentheses are the standard deviations of the measurements. [b] Final GdnHCl concentration at which the refolding step of the double-jump experiment is carried out. [c] The time constants for the CD measurements are the results of a single exponential fit to the refolding decay curves shown in Figures 2 and 3. The time constants for the absorbance measurements were obtained from the data of Houry et al. (1995).

CAM-RNase A is used as a model for unfolded RNase A at low denaturant concentration. It is observed from Table 2 that RNase A and CAM-RNase A give the same CD(222 nm) value at 5.9 M GdnHCl, pH 7, and 5 °C where RNase A is thought to be completely unfolded. However, the CD for CAM-RNase A becomes more negative as the GdnHCl concentration decreases. The significance of this behavior is discussed below.

Table 2: CD Values Obtained from Kinetic and Steady-State Measurements, in (deg·cm^2/dmol)[a]

CD value of	[GdnHCl] (M)					
	CD (275 nm)		CD (222 nm)			
	0.9	5.9	0.9	2.6	4.2	5.9
intercept at "zero" refolding time from kinetic refolding experiments	−89 (9)	NM[b]	−2900 (222)	−1609 (50)	NM	NM
folded RNase A from kinetic refolding experiments[c]	−241 (2)	NM	−8169 (32)	−7999 (24)	NM	NM
RNase A from steady-state measurements[d]	−252 (4)	−59 (5)	−8291 (121)	−8076 (121)	NM	−1149 (83)
CAM-RNase A from steady-state measurements[e]	NM	NM	−2760 (126)	−1843 (85)	−1375 (85)	−1187 (81)

[a] The CD values obtained from kinetic and steady-state measurements are listed in this table. The experimental procedures for each method are given in Materials and Methods. All experiments were carried out at pH 7.0, 5 °C, and the indicated GdnHCl concentration. The numbers in parentheses are the standard deviations of the measurements. [b] NM, not measured. [c] This refers to the value obtained from the kinetic refolding curves when the native state is reached, i.e., at infinite refolding time. [d] This is equivalent to the state obtained in the kinetic experiments at infinite refolding times. [e] This provides the CD values of the reduced protein in the absence of disulfide bonds.

DISCUSSION

In our previous study, we monitored the refolding process of U_{vf} by absorbance over a wide range of pH and GdnHCl concentrations (Houry et al., 1995). From the kinetic data, it was inferred that a kinetic intermediate, I_Φ, exists on the refolding pathway of U_{vf}. However, the presence of this intermediate was not observed directly as a kinetic burst phase by absorbance. In this study, we have attempted to provide direct evidence for the presence of the intermediate by monitoring the refolding process of U_{vf} by CD to see whether a burst phase could be observed in the dead time of the instrument. In addition, CD can provide an estimate of the extent of the structure formed in this intermediate. For this purpose, the refolding of U_{vf} was carried out under two different conditions: one at high GdnHCl concentration (2.6 M) where no significant amount of I_Φ is expected to be present, and another at low GdnHCl concentration (0.9 M) where I_Φ is expected to be highly populated.

In order to determine whether there is a burst phase in the refolding kinetic experiments, the intercept at zero refolding time should be compared to the CD value of U_{vf} under the *refolding* condition employed. A burst phase is defined as the change in the CD value within the dead time of the instrument when compared to the CD of U_{vf}. However, the value of the CD of U_{vf} can be determined only at high GdnHCl concentration and then extrapolated to lower GdnHCl concentrations where the refolding is carried out. Experimentally, the CD values of U_{eq} are first measured at 5.9 M GdnHCl at 222 and 275 nm. Since, from the GdnHCl-dependence curves (Fig. 1 insets), the linear fit to the post-transition region gives a straight line which is almost horizontal at both 222 and 275 nm, we can assume that the extrapolated CD values for U_{eq} at 0.9 and 2.6 M GdnHCl, where the kinetic refolding experiments are carried out, are the same as those obtained from steady-state measurements at 5.9 M GdnHCl for both wavelengths. The CD values obtained for U_{eq} are then used for U_{vf} because, in the Results, we have shown that U_{eq} has the same CD as U_{vf}. Hence, we will assume that the CD value for U_{vf} at 222 nm is −1149 (±83) deg·cm^2/dmol and at 275 nm is −59 (±5) deg·cm^2/dmol (Table 2), independent of GdnHCl concentration between 0.9 and 5.9 M.

Kinetic CD Measurements at High GdnHCl Concentrations. The refolding kinetics of U_{vf} at 2.6 M GdnHCl, pH 7.0, and 5 °C were monitored by CD at 222 nm only (Figure 3A). Under these conditions, the intermediate, I_Φ, exists to less than 2% at zero refolding time [from the data of Houry et al. (1995)].

The time constant obtained from the refolding decay curve as monitored by CD agrees well with the time constant obtained when the refolding of U_{vf} is monitored by absorbance (Table 1). This indicates that both CD (after the burst phase) and absorbance monitor the same refolding event.

At 2.6 M GdnHCl and pH 7.0, the value of the intercept at zero refolding time is −1609 (±50) deg·cm^2/dmol, while the CD value of U_{vf} (or U_{eq}) from the steady-state measurement is −1149 (±83) deg·cm^2/dmol (Table 2). This reveals that the CD value of the intercept at zero refolding time is different, within the indicated experimental errors, from that of U_{vf}. The difference between the two CD values is small [−460 (±97) deg·cm^2/dmol]. This difference might not be of much significance because other sources of systematic errors are not accounted for by the above uncertainties, including the assumption that the CD value of U_{vf} at 2.6 M GdnHCl is exactly the same as that at 5.9 M GdnHCl.

If the burst phase observed for the refolding U_{vf} at 2.6 M GdnHCl is real, this would imply the presence of an intermediate that forms within the dead time of the instrument even at 2.6 M where I_Φ is not expected to be present. Because of the relatively small magnitude of the burst, it is unlikely that this new intermediate has any substantial secondary structure. It is reasonable to assume that it is largely unfolded with no significant buried surface area, and that it is populated prior to the formation of the hydrophobically collapsed intermediate I_Φ. Since it is known that aromatic side chains and disulfides in proteins contribute to the far-UV CD signal (Neubert & Carmack, 1974; Woody, 1978; Kahn, 1979; Hider et al., 1988; Manning & Woody, 1989; Chakrabartty et al., 1993), the small difference in the CD [−460 (±97) deg·cm^2/dmol] suggests that U_{vf} and this intermediate might differ only by some local structural rearrangement involving an aromatic side chain or a disulfide bond.

The presence of this intermediate is unlikely to have any effect on our earlier kinetic analysis (Houry et al., 1995) as explained below. A possible representation of the refolding pathway of U_{vf} would be

$$[U_{vf} \overset{K_i}{\rightleftharpoons} I_U] \overset{K}{\rightleftharpoons} I_\Phi \overset{k}{\rightarrow} N \qquad (3)$$

where I_U is the largely unfolded intermediate that is committed to folding, and I_Φ is the hydrophobically collapsed intermediate. The equilibrium constants are given as $K_i = [I_U]/[U_{vf}]$ and $K = [I_\Phi]/[I_U]$. k is the rate constant for the formation of the native state, N, from I_Φ. The brackets in the above equation are used to indicate that I_U and U_{vf} are

structurally very similar. It should be pointed out that, although we show I_U and I_Φ as being on the refolding pathway of U_{vf}, this need not be the case, and the two intermediates can be off-pathway (Houry et al., 1995). As pointed out by Houry et al. (1995), in the case of marginally stable intermediates, it has not yet been possible to "prove" whether intermediates are on or off the refolding pathway (Baldwin, 1996).

From eq 3, the experimentally determined rate constant for the formation of N from all the other species, is given by

$$k_{vf} = \frac{kKK_i}{1 + K_i + KK_i} \quad (4)$$

where k_{vf} is the experimentally determined rate constant (or the apparent rate constant). Since I_U forms from U_{vf} in the burst phase even at 2.6 M GdnHCl, and since K_i has very little GdnHCl dependence because I_U is a largely unfolded intermediate, it is reasonable to assume that $K_i \gg 1$ under all refolding conditions employed independent of GdnHCl concentration (between 0.9 and 2.6 M).[2] Thus k_{vf} reduces to

$$k_{vf} = \frac{kK}{1 + K} \quad (5)$$

which is the same expression used in our previous study [eq 3 in Houry et al. (1995)]. The values obtained for K and k in our previous study can be used in the current study.

Based on the above discussion, since I_U is a largely unfolded intermediate and, consequently, since K_i has little dependence on GdnHCl concentration, the CD(222 nm) of I_U at 5 °C and pH 7.0 can be taken to be −1609 (±50) deg·cm^2/dmol independent of GdnHCl concentration used (between 2.6 and 0.9 M). This implies that I_U has <7% (460/6927) of the secondary structure present in the native state. Furthermore, the CD of I_U at 275 nm is expected to be the same as that of U_{vf} (i.e., −59 (±5) deg·cm^2/dmol, Table 2) since both I_U and U_{vf} probably do not contain any significant tertiary contacts.

Kinetic CD Measurements at Low GdnHCl Concentration. The refolding kinetics of U_{vf} at 0.9 M GdnHCl, pH 7, and 5 °C were monitored by CD at 275 nm (Figure 2) and at 222 nm (Figure 3B). In both curves, the CD value of U_{vf} is also shown.

The time constants obtained from both curves are similar, and they agree with the time constant obtained from absorbance data (Table 1). Hence, the different monitoring methods follow the same refolding process, which implies that there are no intermediates that are significantly populated after the initial burst phase.

A burst phase was observed at 222 nm and at 275 nm (Figures 2 and 3B). The magnitude of the burst is the difference between the CD of U_{vf} and that of the intercept of the decay curve. The presence of such a burst phase is direct evidence for the presence of an intermediate.

At 222 nm and 0.9 M GdnHCl, the CD value of the intercept of the decay curve at "zero" refolding time is −2900 (±222) deg·cm^2/dmol. From our previous studies, the equilibrium constant between U_{vf} and I_Φ at 0.9 M GdnHCl, pH 7.0, and 5 °C, based on eq 1, was found to be 0.66 (Houry et al., 1995). In light of the new model for the refolding of U_{vf} (eq 3), the equilibrium constant can be considered to be between I_U and I_Φ. Furthermore, since $K_i \gg 1$, we can assume that, just after the initiation of folding, there is about 0% U_{vf}, and the value of $K = 0.66$ implies that there is about 60% I_U and 40% I_Φ. Since the CD values of both U_{vf} and I_U are assumed to be independent of GdnHCl concentration, then, at 222 nm and 0.9 M GdnHCl, the CD value of U_{vf} is taken to be −1149 (±83) deg·cm^2/dmol and that of I_U to be −1609 (±50) deg·cm^2/dmol (Table 2). Therefore, the calculated CD(222 nm) of I_Φ is −4837 (±560) deg·cm^2/dmol at pH 7.0 and 5 °C [from $0.6(-1609) + 0.4\mathrm{CD}(I_\Phi) = -2900$].

At 275 nm and 0.9 M GdnHCl, a burst phase can also be observed by CD (Fig. 2). The intercept from the kinetic experiment at zero refolding time is −89 (±9) deg·cm^2/dmol, while the value for U_{vf}, from steady-state measurement, is −59 (±5) deg·cm^2/dmol. If we assume that I_U has the same CD value as U_{vf} at 275 nm, then, using the same assumptions and the same mathematical calculation as in the previous paragraph, the calculated CD(275 nm) of I_Φ would be −134 (±24) deg·cm^2/dmol at pH 7.0 and 5 °C.

The CD kinetic experiments provide direct evidence for the presence of I_Φ in the form of a burst phase. In addition, an estimate of the amount of structure formed in I_Φ can be obtained. From the CD values at 222 nm, we can say that I_Φ has about 52% of the secondary structure which is present in the native state [from (4837 − 1149)/(8291 − 1149)]. However, this secondary structure need not be due only to helix formation since other groups contribute to the CD at 222 nm. Furthermore, the secondary structure present in I_Φ can be highly dynamic so that it might not be detectable by other methods, such as H−D exchange (Guijarro et al., 1995; Gryk & Jardetzky, 1996). From the CD values at 275 nm, we can estimate that I_Φ contains about 39% of the tertiary contacts that are present in the native state [from (134 − 59)/(252 − 59)]. There is a contribution from the tyrosines as well as from the disulfides to the near-UV CD of RNase A (Horwitz et al., 1970; Takagi & Izutsu, 1974). Since we do not see any burst phase by absorbance for the formation of I_Φ, this suggests that the exposed tyrosines, rather than the buried ones, contribute to the burst-phase change in the CD at 275 nm. In native RNase A, two of these exposed, or partly exposed, tyrosines are part of type VI β-bends and are located next to prolines, namely, Tyr 92-Pro 93-Asn 94 and Asn 113-Pro 114-Tyr 115. The X-Pro peptide bonds in both turns are in the *cis* conformation in the native state. In peptides containing the sequence X-Pro-Tyr, Kemmink and Creighton (1995) have shown the presence of relatively strong local interactions between the aromatic ring and the side chain of the proline only when the X-Pro peptide bond is *cis*. Furthermore, Dyson et al. (1988) have shown that, in peptides of the form Tyr-Pro-Y-Asp-Val, a high population of structured forms are present in these peptides, especially of type VI β-turns, when the Tyr-Pro peptide bond is in the *cis* conformation. Therefore, the CD obtained for I_Φ at 275

[2] Whether or not we assume that K_i is much greater than 1 does *not* change the CD values calculated for I_Φ later in the Discussion. If K_i is not much greater than 1, then the equilibrium constant obtained in our previous study (Houry et al., 1995), in light of eq 3 and 4, would be equal to $[I_\Phi]/([I_U] + [U_{vf}])$ instead of $[I_\Phi]/[U_{vf}]$. The CD values calculated for I_Φ at 222 and 275 nm using the new equilibrium expression will be the same as those given in the subsequent section provided that K_i has little GdnHCl dependence. Only the estimated CD value of I_U will be affected.

nm might be due to the formation of these β-turns once the GdnHCl is diluted. In the native structure of RNase A, the type VI β-bends, involving Tyr 92 and Tyr 115, are important for the formation of β-sheets in the protein. Hence, the CD signal obtained at 275 nm could also imply the possible formation of sheet structures in I_Φ. Alternatively, the near-UV CD signal might be due to the isomerization or the change in the environment of one of the disulfides in the protein. In summary, on the basis of the CD evidence and of our previous study (Houry et al., 1995), we can characterize I_Φ as being a hydrophobically collapsed intermediate containing significant secondary and tertiary structure.

CD of CAM-RNase A. In order to investigate the structure present in the largely unfolded intermediate I_U further, CAM-RNase A was used as a model polypeptide. We have argued that I_U does not have any significant buried surface area and that the structure present in I_U is most probably local, arising from specific interactions between neighboring amino acid residues involving aromatic side chains or disulfide bonds, or from hydrophobic clustering in small segments of the protein. Hence, we expect that the structure present in CAM-RNase A at 2.6 M GdnHCl, pH 7.0, and 5 °C to be similar to whatever structure is present in I_U under the same conditions. In other words, we expect the CD of I_U and CAM-RNase A to be similar under these conditions. To this end, we have carried out steady-state CD measurements on CAM-RNase A at different GdnHCl concentrations at pH 7.0 and 5 °C.

It is observed that, as the GdnHCl concentration is lowered, the CD of CAM-RNase A becomes more negative (Table 2). Similar behavior was observed for CAM-RNase A at pH 2.0 and for completely reduced and sulfonated ribonuclease A at pH 7.0 (data not shown). NMR studies on peptide fragments corresponding to the amino terminal sequence of RNase A (Silverman et al., 1972; Kim & Baldwin, 1984; Osterhout et al., 1989) indicate that these fragments can adopt a helical conformation. Also, NMR studies on reduced S-sulfonated RNase A (Swadesh et al., 1984) indicate the presence of local structure around His 12 (which is part of the first helix in the native protein) at pH 3.0 and temperatures below 35 °C. Chavez and Scheraga (1980) used antibodies specific for native antigenic determinants in the protein to show that reduced and reduced S-carboxymethylated ribonuclease A retain native-like structures at 4 °C and pH 8.3. Finally, the study by Buckler et al. (1995), using time-resolved nonradiative fluorescence energy transfer, indicates that reduced ribonuclease A in 0.6 M GdnHCl, pH 5.0, and 22 °C is more compact than reduced ribonuclease A in 6.7 M GdnHCl at the same pH and temperature, especially in the C-terminal segment. Therefore, on the basis of the above evidence from the literature, we attribute the decrease in the CD signal of CAM-RNase A with decreasing GdnHCl concentration at pH 7.0 and 5 °C to the formation of nonrandom structure in the reduced protein.

At 5.9 M GdnHCl, pH 7.0, and 5 °C, it is observed that both CAM-RNase A and RNase A have the same CD signal at 222 nm. This is a good indication that both proteins have similar structures under these conditions and that these structures can be described as statistical coils. On the other hand, at 2.6 M GdnHCl, the CD of CAM-RNase A [-1843 (± 85) deg·cm^2/dmol] is similar to that of I_U [-1609 (± 50) deg·cm^2/dmol, the value obtained for the burst phase]. This is consistent with our previous assertion that the structure present in I_U is probably local in nature and does not result in significant burial of surface area. The slight difference between the two values might be due to the fact that the comparison is being made between a cross-linked and a non-cross-linked protein. Unfortunately, no meaningful comparison can be made between RNase A and CAM-RNase A at 0.9 M GdnHCl. Under this condition, I_Φ, which has significant buried surface area, becomes populated in the burst phase (in addition to I_U). Consequently, the presence or absence of the disulfide cross-link will play a major role in the formation of structure. The agreement of the CD value of CAM-RNase A [-2760 (± 126) deg·cm^2/dmol] and of the intercept at "zero" refolding time [-2900 (± 222) deg·cm^2/dmol] at 0.9 M GdnHCl is only coincidental.

Further Evidence for the Presence of I_U. In a recent study of a Tyr 92 to Trp (Y92W) mutant of RNase A, a burst phase was observed by fluorescence for the refolding of U_{vf} at 2.5 M GdnHCl, pH 5.0, and 10 °C (R. A. Sendak, D. M. Rothwarf, W. J. Wedemeyer, W. A. Houry, and H. A. Scheraga, submitted for publication). This provides further support for the presence of I_U on the refolding pathway of U_{vf}. It suggests that the type VI β-turn containing Tyr 92 is involved in the local structural rearrangement that results in the formation of I_U.

CONCLUSION

The refolding pathway of U_{vf} has been investigated by CD spectroscopy. The data indicate the presence of a largely unfolded intermediate, I_U, that forms directly from U_{vf}, and that I_U then undergoes hydrophobic collapse resulting in the formation of the molten-globule-like intermediate I_Φ. The structure present in I_U is most probably of a local nature with no significant burial of surface area. On the other hand, I_Φ has a large CD signal at 222 and 275 nm on the order of 52% and 39% that of native RNase A, respectively. This suggests the presence of significant secondary structure and tertiary contacts in this intermediate.

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