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Cis proline mutants of ribonuclease A. II. Elimination of the slow-folding forms by mutation

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Abstract

Ribonuclease A is known to form an equilibrium mixture of fast-folding (U_F) and slow-folding (U_S) species. Rapid unfolding to U_F is then followed by a reaction in the unfolded state, which produces a mixture of U_F, U_SII, U_SI, and possibly also minor populations of other U_S species. The two cis proline residues, P93 and P114, are logical candidates for producing the major U_S species after unfolding, by slow cis ⇔ trans isomerization. Much work has been done in the past on testing this proposal, but the results have been controversial. Site-directed mutagenesis is used here. Four single mutants, P93A, P93S, P114A, and P114G, and also the double mutant P93A, P114G have been made and tested for the formation of U_S species after unfolding. The single mutants P114G and P114A still show slow isomerization reactions after unfolding that produce U_S species; thus, Pro 114 is not required for the formation of at least one of the major U_S species of ribonuclease A. Both the refolding kinetics and the isomerization kinetics after unfolding of the Pro 93 single mutants are unexpectedly complex, possibly because the substituted amino acid forms a cis peptide bond, which should undergo cis → trans isomerization after unfolding. The kinetics of peptide bond isomerization are not understood at present and the Pro 93 single mutants cannot be used yet to investigate the role of Pro 93 in forming the U_S species of ribonuclease A.

The double mutant P93A, P114G shows single exponential kinetics measured by CD, and it shows no evidence of isomerization after unfolding. Thus, the unfolding and refolding kinetics of the double mutant indicate that replacement of Pro 93 and Pro 114 has removed the residues responsible for forming the major U_S species. This result, taken together with data for the Pro 114 single mutants, indicates that Pro 93 and Pro 114 are collectively responsible for forming the major U_S species.

Keywords: cis proline mutants; folding/unfolding kinetics; ribonuclease A; slow-folding forms

The existence of an equilibrium mixture of fast-folding (U_F) and slow-folding (U_S) forms of an unfolded protein was discovered in RNase A (Garel & Baldwin, 1973), and later proved to be a common property of many proteins, and was the source of the proline isomerization model of Brandts et al. (1975). The U_S species account for most of unfolded RNase A (U_S:U_F = 80:20; Garel & Baldwin, 1973; Schmid, 1982) and the two cis proline residues, Pro 93 and Pro 114 (Kinemage 1), provide logical candidates for producing these U_S species, as the trans isomer of a proline residue is expected to be the major form when the protein is unfolded. Typical trans:cis ratios of proline isomers in unstructured peptides are 80:20 or 90:10 (Brandts et al., 1975; Grathwohl & Wüthrich, 1976a,b, 1981).

The proline isomerization model of Brandts et al. (1975) postulates that the refolding pathway of any protein is

$$U_S \overset{isom}{\Leftrightarrow} U_F \overset{fold}{\rightarrow} N, \qquad (1)$$

where N is the native protein and folding intermediates are not populated even transiently. The model proposes that the slow-folding property of a U_S species is explained by slow proline isomerization ($U_S \rightarrow U_F$) at the first step of U_S refolding. The major U_S species of RNase A (U_SII) was found, however, to fold in strongly native conditions by a pathway in which folding precedes

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isomerization (Cook et al., 1979; Schmid & Blaschek, 1981):

$$U_S II \xrightarrow{fold} I_N \xrightarrow{isom} N. \quad (2)$$

The intermediate I_N is native-like in most physical properties, including the specific binding of the substrate analog 2′CMP (Cook et al., 1979; Schmid & Blaschek, 1981) and I_N has RNase catalytic activity (Schmid & Blaschek, 1981). Proof that the folded species I_N is formed before isomerization occurs is provided by a direct assay based on first unfolding, then refolding, the sample: I_N unfolds to give U_S, whereas N unfolds to give U_F (Cook et al., 1979). Tyrosine fluorescence can be used to monitor the $U_F \Leftrightarrow U_S$ reaction in unfolded RNase A (Rehage & Schmid, 1982) and Tyr 92, which is adjacent to the cis proline residue P93 (Kinemage 3), contributes strongly to the observed signal (Schmid et al., 1986).

Several approaches have been tried to find out if Pro 93 and/or Pro 114 account for the U_S species of unfolded RNase A (see Discussion). An approach that should be straightforward but which has yielded contradictory results is to measure the trans:cis isomer ratios of Pro 93 and Pro 114 in unfolded RNase A, to find out if either proline residue or both residues can account for the relative proportions of the U_SII and U_SI species: U_F:U_SII:U_SI = 20:60–70:20–10 (Schmid, 1983). (An additional minor U_S species has been reported by Lin & Brandts [1987].) Results of isomer-specific proteolysis (Lin & Brandts, 1983, 1984) indicate that neither P93 nor P114 shows a large enough amount of the wrong trans isomer to account for U_SII: the trans:cis ratios of Pro 93 and Pro 114 in urea-unfolded RNase A are reported by Lin and Brandts (1984) to be 30:70 and 5:95, respectively. On the other hand, Adler and Scheraga (1990), who used ^{1}H-NMR, found that the trans:cis percentages of Pro 93 and of Pro 114 in heat-unfolded RNase A are about 60:40 and 63:37, respectively, so that either proline residue might by itself account for U_SII. The reason for the difference between the results of Lin and Brandts (1984) and of Adler and Scheraga (1990) remains to be discovered.

Site-directed mutagenesis is used here to find out if the cis proline residues of RNase A produce the U_S species. Our procedure is to analyze the $U_F \Leftrightarrow U_S$ isomerization process in the unfolded protein, rather than to analyze the changes produced by mutation in the refolding kinetics. Folding intermediates can introduce new kinetic phases in refolding; moreover, replacement of a cis proline residue can cause major changes in the overall refolding kinetics of a protein, as we report here for replacement of Pro 93. Consequently, the simple experiment of asking whether one slow phase in refolding is eliminated by mutating a proline residue is not necessarily a good way to proceed. On the other hand, replacement of one proline residue should have little effect on the cis–trans isomerization of the remaining proline residues in the unfolded protein, provided they are distant in sequence from the mutated residue, so that analysis of the $U_F \Leftrightarrow U_S$ reaction in the unfolded protein is expected to be straightforward. The presence of a slow isomerization reaction after unfolding is the most direct test for the existence of separate U_F and U_S forms.

In the preceding paper, we show that single mutants in each cis proline residue of RNase A, and also a double mutant, can be expressed and refolded to give catalytically active protein, and so the problem is open to study.

Results

Refolding kinetics of single mutants at P114 and P93

To find conditions for detecting slow isomerization reactions occurring in an unfolded protein, the first step is to compare the refolding kinetics immediately after unfolding (protein labeled U_0) with those found later, after unfolding equilibrium has been reached (protein labeled U_∞). If the protein unfolds and isomerizes by a simple $N \rightarrow U_F \Leftrightarrow U_S$ mechanism, U_0 can be identified with U_F and U_∞ should contain an equilibrium mixture of U_F and U_S. Then, by measuring the refolding kinetics of U_0 and U_∞ in different conditions, one can find optimal conditions for distinguishing U_F from U_S. For bovine RNase A, this procedure gives straightforward results (Brandts et al., 1975) although the experiments are complicated by the presence of at least two different U_S species.

The U_F species of bovine RNase A refolds rapidly, in a single exponential time course, when measured by probes of tertiary structure, such as tyrosine absorbance of 2′CMP binding. Provided the denaturant concentration is low, the time range of refolding is 10–100 ms over a wide range of pH and temperature (Garel et al., 1976; Hagerman & Baldwin, 1976; Lin & Brandts, 1983). The logarithm of the refolding rate decreases linearly with denaturant concentration: there is roughly a 1,000-fold drop between zero denaturant and the edge of the unfolding transition (Nall et al., 1978; Tsong & Baldwin, 1978). Only the U_S species of bovine RNase A refold slowly enough to be measured in manual mixing experiments, which we use here. Mutant proteins, on the other hand, may refold more slowly than wild type, and the U_0 forms of some mutants studied do show measurable refolding kinetics in manual mixing experiments.

A typical refolding experiment is illustrated in Figure 1. CD at 222 nm, the usual wavelength for observing α-helix formation, is used to monitor refolding in this experiment. The CD spectra of bovine RNase A and these mutants are similar (Schultz & Baldwin, 1992) and a low protein concentration, in the range 1–10 μm, can be used, which minimizes problems of aggregation. Figure 1A shows a kinetic trace for refolding of the U_0 form of the

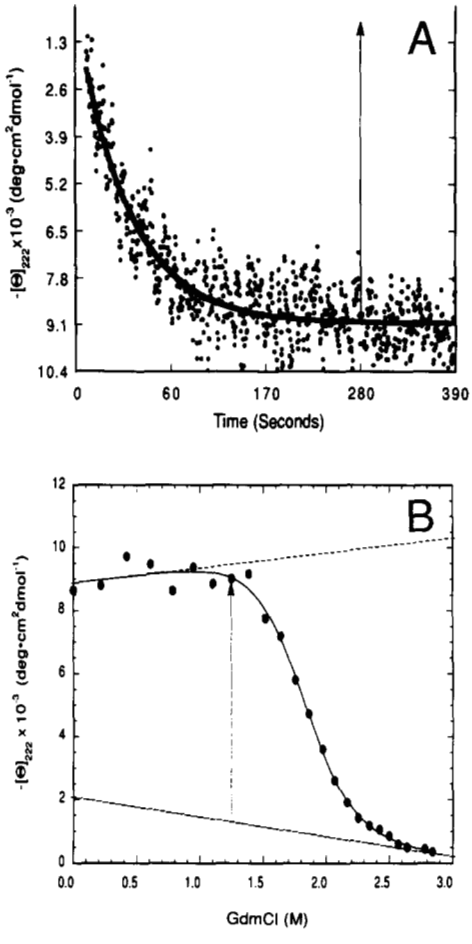

Fig. 1. **A**: Kinetic trace for refolding of the double mutant P93A, P114G measured by CD. Conditions: pH 6.0, 0.05 M Na cacodylate, 1.25 M GdmCl, 4 μM protein, 0 °C. The kinetic amplitude (see arrow) is compared to the expected total amplitude from the GdmCl-induced unfolding curve (Fig. 1B). **B**: Equilibrium transition curve for GdmCl-induced unfolding of the double mutant P93A, P114G. Conditions: as in Figure 1A, except 15 μM protein.

double mutant P93A, P114G; measurement of the observed kinetic amplitude is indicated by an arrow. The equilibrium transition curve of the double mutant, for unfolding induced by GdmCl, is shown in Figure 1B, and measurement of the expected total kinetic amplitude is shown by an arrow. In later figures, the term "fractional amplitude" refers to the ratio of the observed kinetic amplitude to the equilibrium amplitude measured in this way. When the fractional amplitude is less than one, part of the kinetic refolding process is too rapid to be measured in manual mixing experiments, either because two species (e.g., U_F, U_S) are present, one of which refolds rapidly, or because the refolding kinetics of a single species are complex, and one or more kinetic intermediates are formed within the dead time of mixing.

For single mutants with a mutation in P114, only the folding kinetics of the U_S species are slow enough to measure in manual mixing experiments at GdmCl concentrations below the unfolding transition zone (≤1 M GdmCl for P114G, 10 °C, pH 6.0). Even at higher GdmCl concentrations inside the unfolding transition zone, the U_0 forms of P114G and P114A refold too rapidly to be measured by manual mixing. The refolding kinetics of the U_∞ forms of P114G and P114A are shown in Figure 2, plotted against GdmCl concentration: the refolding rate ($1/\tau$) is shown in Figure 2A, and the fractional amplitude is shown in Figure 2B.

The refolding kinetics of single mutants with a mutation in P93 are decidedly different. Even the U_0 forms show complex folding kinetics, with a slow phase and at least one fast phase detected by CD at GdmCl concentrations above 1 M, and with a very slow phase in refolding detected either by tyrosine fluorescence or by unfolding rate assays. Figure 3 illustrates some features of this complex refolding behavior; a complete study of the refolding mechanism of P93 mutants will be left for future work. Figure 3A shows the conversion of P93A from U_0

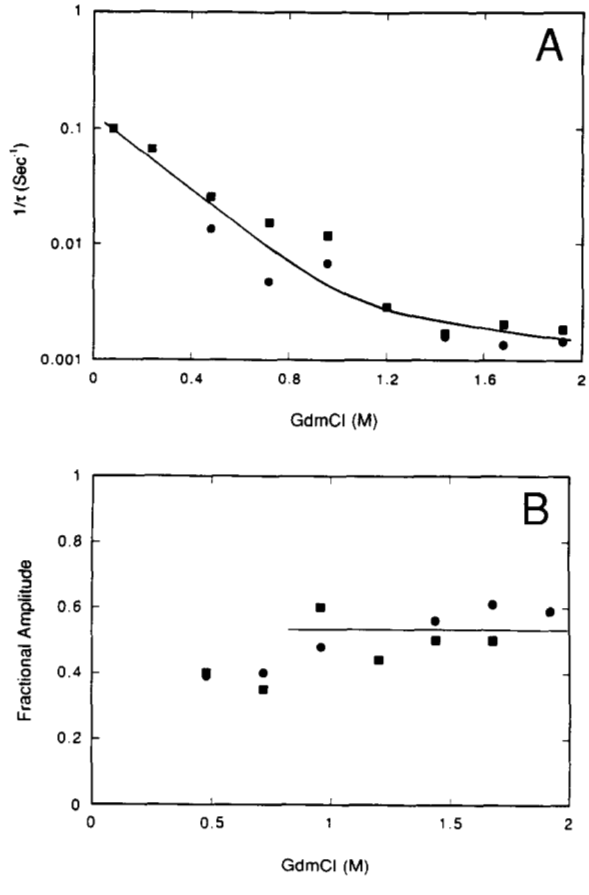

Fig. 2. Folding kinetics of two P114 single mutants versus GdmCl concentration at pH 6.0, 0.05 M Na cacodylate, 10 °C, 5 μM protein (●, P114G; ■, P114A). The properties of the equilibrium unfolded protein (U_∞ form) are shown: only the U_S species folds slowly enough to be observed. Folding is measured by CD as in Figure 1A. The folding rate ($1/\tau$) is shown in **A** and the fractional amplitude observed in the kinetic experiment is shown in **B**. See legend to Figure 1A.

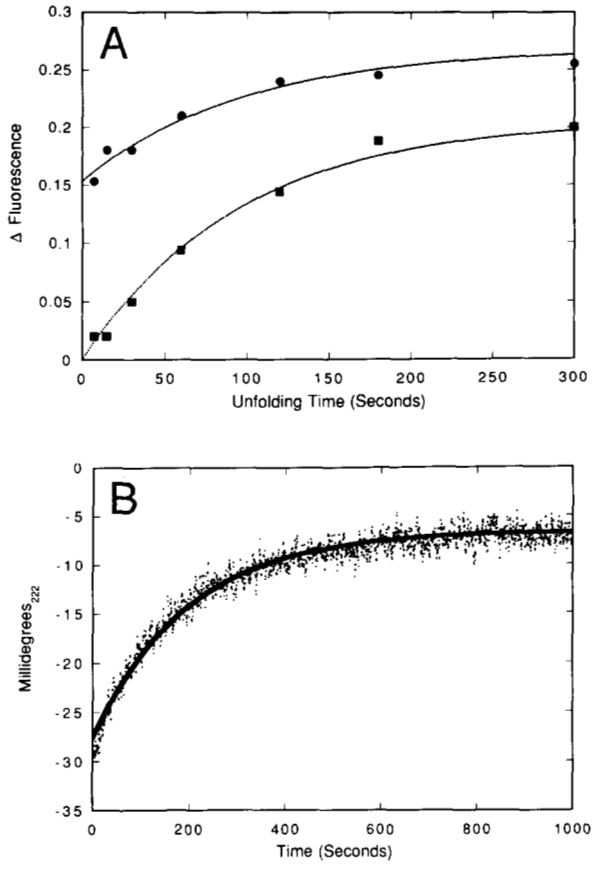

Fig. 3. Some properties of the refolding kinetics of P93A. **A**: Refolding assays, monitored by fluorescence, versus time after unfolding in 4 M GdmCl, 0.05 M glycine, pH 2, 10 °C, 60 μM protein. Refolding conditions: 1.0 M GdmCl, 0.05 M Na cacodylate, pH 6, 11 °C, 3 μM protein. Fluorescence measurements: excitation 268 nm, band width 5 nm; emission 305 nm, band width 10 nm. The amplitudes of two kinetic phases in refolding are shown, and a third, faster phase is not shown; the relaxation times of the two phases are: ●, τ_1, 800 s; ■, τ_2, 60 s. The P93A mutant protein studied in this experiment was expressed in *B. subtilis*. **B**: Kinetic unfolding trace of the product formed when U_0 refolds for 60 s in 0.4 M GdmCl, 0.05 M Na cacodylate, pH 6.0, 10 °C. The unfolding conditions are: 3 M GdmCl, 0.05 M Na cacodylate, pH 6.0, 10 °C. The unfolding trace is fitted by a single exponential whose relaxation time is 200 s. In the same conditions, native P93A unfolds with a relaxation time of 500 s.

to U_∞ versus time after unfolding, with refolding monitored by tyrosine fluorescence. A very slow phase (τ_1 = 800 s) is observed in the refolding of U_0 and its amplitude increases as U_∞ is formed. Thus, U_0 is not a straightforward fast-folding species. Moreover, an isomerization reaction can be detected with time after unfolding when refolding is monitored by fluorescence or CD (data not shown). A second midrange phase in refolding (τ_2 = 60 s) is not observed when U_0 refolds but is observed in the refolding of U_∞ and thus is suitable for monitoring isomerization after unfolding. A third faster phase (τ_3 = 10 s) is not well resolved and cannot be quantitated. Figure 3B shows the unfolding kinetics of the product formed after 60 s refolding when U_0 refolds at 0.4 M GdmCl, conditions in which CD-detected refolding is rapid. The unfolding kinetics of the product are slow enough to be measured in a manual mixing experiment, which suggests that the product is either native or native-like. The unfolding rate is about 2.5 times faster than that of the native protein (see legend to Fig. 3B). Other experiments (not shown) indicate that conversion from the faster unfolding rate shown in Figure 3B to the native unfolding rate occurs in the very slow phase of refolding (τ_1 = 800 s) that is also detected by fluorescence (Fig. 3A). Another single mutant with a P93 mutation, P93S, has been studied: it also shows complex refolding kinetics like those of P93A.

Because the isomerization reactions after unfolding, as well as the refolding kinetics, of single mutants with a mutation in P93 are very different from those with a mutation in P114 and from those of bovine RNase A, it is premature to draw conclusions from the behavior of P93A or P93S about slow-folding species caused by the isomerization of Pro 114 in bovine RNase A, and no additional data are presented here concerning isomerization reactions in unfolded P93A or P93S.

Isomerization after unfolding of the single mutant proteins

Two mutant proteins with single mutations in P114 have been tested for isomerization reactions after unfolding. The time course of isomerization after unfolding of P114G and P114A is given in Figure 4; the ordinate shows the amount of slow-folding material at each time after unfolding. The rate of folding measured in each refolding assay shows no measurable change with time after un-

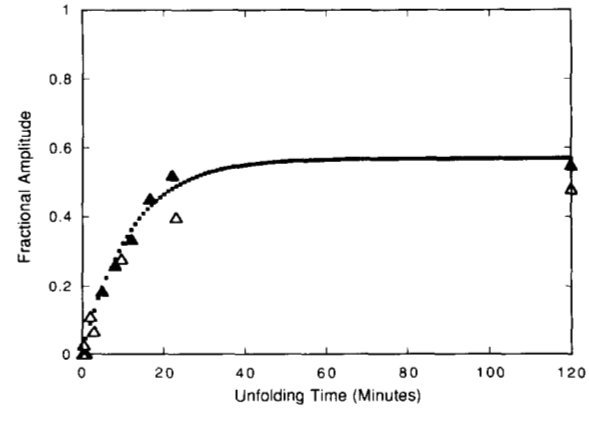

Fig. 4. Isomerization after unfolding of P114G and P114A. Fractional amplitude (see legend to Fig. 1A) of species refolding slowly: △, P114G; ▲, P114A. Refolding assays are used to monitor isomerization; only the U_S species refold(s) slowly enough to be observed in these refolding assays. Conditions of unfolding: 4 M GdmCl, 0.05 M glycine, pH 2.0, 0 °C; conditions of refolding: 1.5 M GdmCl, 0.05 M Na cacodylate, pH 6.0, 10 °C.

folding (data not shown). The isomerization reactions of P114G and P114A are similar both as regards their amplitudes (Fig. 4) and refolding rates. Thus, isomerization after unfolding fits a simple $N \rightarrow U_F \Leftrightarrow U_S$ unfolding mechanism. The cis → trans isomerization of Pro 93 after unfolding is a possible candidate for producing the $U_F \Leftrightarrow U_S$ reaction shown in Figure 4.

Refolding kinetics and isomerization behavior of the double mutant P93A, P114G

The CD-detected refolding kinetics of the double mutant P93A, P114G are remarkably simple. Despite the fact that the P93A mutation is present, both the U_0 and U_∞ forms refold with a single exponential time course, and the kinetics are identical for both forms at all GdmCl concentrations. Figure 5 compares the time course of refolding of U_0 and U_∞ at one GdmCl concentration, and Figure 6 compares their refolding rates (Fig. 6A) and fractional amplitudes (Fig. 6B) at a series of GdmCl concentrations. The fractional amplitude is 1.0 ± 0.1 in all cases: the observed kinetic curve actually accounts for the entire amplitude expected from the equilibrium unfolding transition (see Fig. 1). Consequently, no difference between U_0 and U_∞ can be detected in the double mutant by the same techniques used to study isomerization after unfolding of the P114 single mutants; thus, the unfolding kinetics of the double mutant fit the simple mechanism $N \rightarrow U_F$.

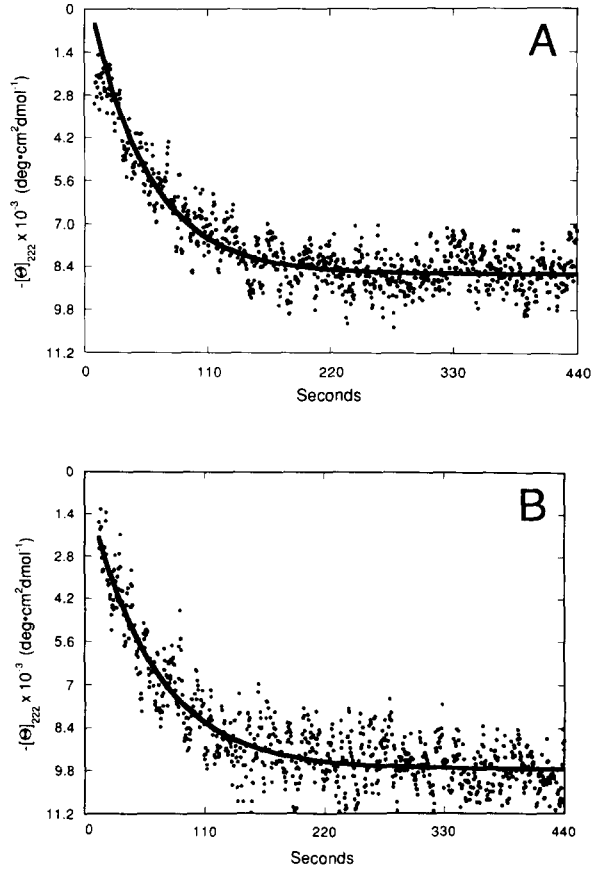

Fig. 5. Comparison between the refolding kinetics of the U_0 and U_∞ forms of the double mutant P93A, P114G in one set of conditions. Refolding conditions: 1.25 M GdmCl, 0.05 M Na cacodylate, pH 6.0, 0 °C. Unfolding conditions: 4.0 M GdmCl, 0.05 M glycine, pH 2.0, 0 °C. **A**: Equilibrium unfolded protein (U_∞ form, produced by 2 h unfolding). **B**: U_0 form (produced by 30 s unfolding).

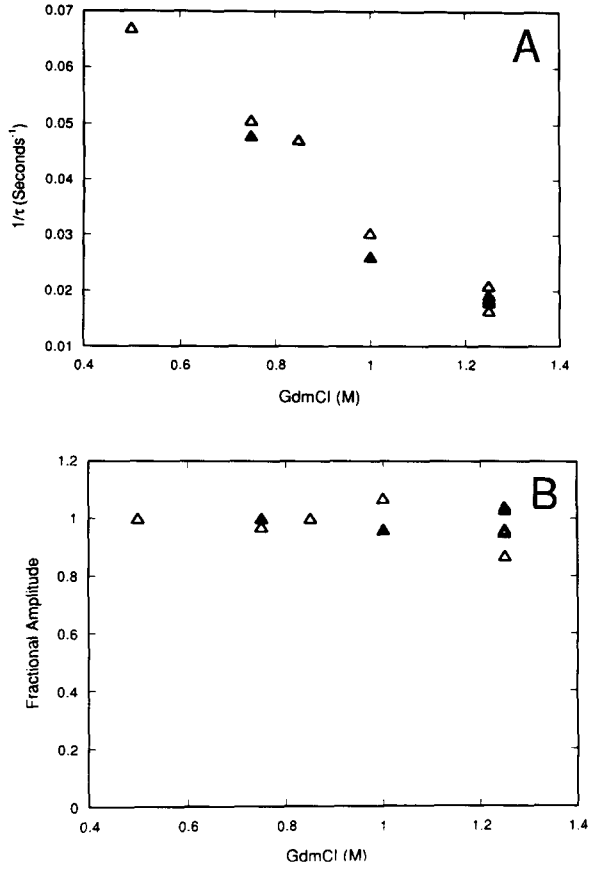

Fig. 6. Comparison between the refolding kinetics of the U_0 and U_∞ forms of the double mutant P93A, P114G at several GdmCl concentrations (0.05 M Na cacodylate, pH 6.0, 10 °C): △, U_0; ▲, U_∞. **A**: Refolding rate. **B**: Fractional amplitude observed in refolding (see legend to Fig. 1A).

Discussion

Origin of the major U_S species of bovine RNase A

The double mutant results indicate that replacement of Pro 93 and Pro 114 is sufficient to eliminate the major U_S species of bovine RNase A. Because isomerization still occurs in the Pro 114 single mutants, Pro 114 is not solely responsible for forming the U_S species. Unfortunately, the Pro 93 single mutant results are not interpretable at this time. Thus, the main conclusion from our

work is that the two cis proline residues are responsible for the major U_S species of RNase A, but it is not yet known how they account for the individual species U_SII and U_SI. The ratio of (U_F):(U_S) is known accurately (0.18:0.82; Schmid, 1982), but the detailed breakdown of the U_S species is not known accurately. Two proline residues are needed to produce two or more U_S species, and it seems likely that both Pro 93 and Pro 114 participate in forming the U_S species of RNase A. In future work, it will be important to use other spectroscopic probes, in addition to CD, to monitor the refolding of the double mutant and of the Pro 114 single mutants; it is particularly important to determine accurately the (U_F):(U_S) ratio in the Pro 114 single mutants.

The U_SII species is known to fold rapidly in strongly native conditions and to form the native-like intermediate I_N before proline isomerization occurs (Cook et al., 1979; Schmid & Blaschek, 1981; Schmid, 1983). Moreover, the fluorescence of Tyr 92 is known to monitor the $U_F \Leftrightarrow U_S$ reaction in unfolded RNase A (Rehage & Schmid, 1982; Schmid et al., 1986). Thus, by a combination of unfolding and refolding assays, it was possible to show that U_SII apparently contains a nonnative trans isomer of Pro 93 (Schmid et al., 1986). This conclusion has, however, been disputed (Lin & Brandts, 1988).

Our results provide a rough estimate of the minimum amount of U_S in GdmCl-unfolded P114 mutants, given by the fractional amplitude of the slow phase in refolding of U_∞ ($0.5_5 \pm 0.1$, see Fig. 4). If there is a hidden fast phase in the refolding of U_S, it will be counted with U_F: thus, the fractional amplitude gives a minimum estimate of U_S. If the U_S form corresponds to unfolded molecules with a trans isomer of P93, then our estimate of the amount of the P93 trans isomer agrees satisfactorily with the NMR value (0.60) of Adler and Scheraga (1990) for heat-unfolded RNase A, and it disagrees with the isomer-specific proteolysis value (0.30) obtained by Lin and Brandts (1984). Biringer and Puntambekar (1991) made the 115-nitrotyrosyl and 115-amino-tyrosyl derivatives of RNase A in order to measure by NMR the trans:cis isomer ratio of the neighboring P114 residue in unfolded RNase A. They report a trans:cis ratio for P114 of 0.45:0.55 in heat-unfolded RNase A and 0.62:0.38 in GdmCl-unfolded RNase A, in fair agreement with the 0.63:0.37 ratio reported by Adler and Scheraga (1990) for heat-unfolded RNase A and in definite disagreement with the 5:95 ratio reported by Lin and Brandts (1984) for urea-unfolded RNase A.

It is puzzling to understand how the amounts of the trans isomers found by Adler and Scheraga (1990) (0.60 for P93 and 0.63 for P114) can explain the amounts of U_SII and U_SI in bovine RNase A, given our finding that P93 and P114 are collectively responsible for forming the major observable U_S species. Possible solutions to the puzzle are discussed by Adler and Scheraga (1990) and more work is needed to resolve it.

Involvement of cis proline residues in slow-folding reactions of proteins

The same logic which implies a priori that P93 and P114 are likely to be involved in the formation of the slow-folding species of RNase A applies also to other proteins with cis proline residues. Kelley and Richards (1987) used site-directed mutagenesis to replace a cis proline residue, Pro 76, with alanine in *E. coli* thioredoxin. They found that the P76A mutant lacks the major, very slow phase in refolding shown by the oxidized wild-type protein and they concluded that the major U_S species of wild type contains the trans isomer of P76. RNase T1 contains two cis proline residues, Pro 39 and Pro 55, and 96% of the unfolded protein is U_S, as measured by isomerization ($U_F \Leftrightarrow U_S$) after unfolding (Kiefhaber et al., 1990a,b). The entire slow-folding reaction is catalyzed by PPI (peptidyl prolyl isomerase), which indicates that the U_S species are produced by cis–trans isomerization of proline residues. Replacement of Pro 55 by Asn, together with replacement of Ser 54 by Gly, eliminates a major U_S species. The Gly–Asn peptide bond of the mutant is believed to be trans. Staphylococcal nuclease contains a proline residue at position 117; cis–trans isomerization of Pro 117 occurs within folded nuclease, giving rise to two alternative folded conformations (Evans et al., 1989). Replacement of Pro 117 by Gly removes a major slow phase in the refolding kinetics of nuclease (Kuwajima et al., 1991).

Several examples are now known in which introduction or replacement of a trans proline residue does not significantly affect the slow refolding kinetics (Ramdas & Nall, 1986; Herning et al., 1991; Chen et al., 1992). Ribonucleases from the red deer and roe deer contain one or two additional proline residues, respectively, and they show the same slow-refolding kinetics as bovine RNase A (Krebs et al., 1983). These could be examples either of nonessential proline residues (ones in which the presence of either a cis or a trans isomer does not affect the refolding kinetics) or of proline residues that undergo negligible trans → cis isomerization after unfolding. They could also include examples of folding to form a native-like intermediate that contains a nonnative isomer: the subsequent proline isomerization reaction after folding occurs may be invisible to spectroscopic probes, but can be detected by the rate of unfolding (see Fig. 3B and Schmid, 1983).

The refolding rate of the double mutant

At first sight, the refolding rate of the double mutant is anomalously low for a U_F species, as it refolds in the time range 10–100 s between 0.4 and 1.3 M GdmCl, pH 6.0, 10 °C (Fig. 6A). These GdmCl concentrations are, however, either just below (at 0.4 M) or within (at 1.3 M) the unfolding transition zone: see Figure 1. The U_F species of bovine RNase A refolds with a relaxation time of

about 10 s at the edge of the unfolding transition zone (3 M GdmCl) at pH 6.0, 10 °C (Kiefhaber & Schmid, 1992), which is close to the value shown by the double mutant at 0.4 M GdmCl. The double mutant is much less stable than wild-type bovine RNase A and its T_m is reduced by 20 °C in thermal unfolding experiments (Schultz & Baldwin, 1992). When 0.6 M GdmSCN is used to lower the T_m of RNase A by about 20 °C, the refolding rate of the U_F species drops about 100-fold at 25 °C (Tsong & Baldwin, 1978, Table III), which shows the strong dependence of the folding rate on the stability of the folded protein.

Materials and methods

See the preceding paper (Schultz et al., 1992) for other information concerning Materials and methods.

GdmCl-induced unfolding curves

GdmCl research grade from Bethesda Research Laboratories was used. Concentrations of stock solutions were determined by refractive index. Unfolding was induced by successive additions of 6 M GdmCl to protein in 0.05 M Na cacodylate, pH 6.0, 0 °C; 10 min was allowed for equilibration after each addition before recording the ellipticity at 222 nm.

Kinetic unfolding and refolding experiments

Standard unfolding conditions are 4 M GdmCl, 0.05 M glycine, pH 2.0, 0 °C. Samples of U_0 and U_∞ are left unfolded for 30 s and 2 h, respectively, before initiating refolding by 50-fold dilution into refolding buffer (0.05 M Na cacodylate, pH. 6.0, 0 °C or 10 °C, varying M GdmCl). Manual mixing employed a small plastic spatula plus magnetic stirring; mixing was complete in 15 s. The CD signal was sampled every 0.5 s and was averaged with a window of 1 s. The kinetic trace was started before initiating refolding, and refolding was allowed to continue for at least four times the relaxation time. Data from the kinetic trace were either fitted directly to an exponential function, using the KaleidaGraph program from Synergy, or else the logarithm of the difference between the CD values at time t and infinite time was plotted against time. The measurement of kinetic and equilibrium amplitudes is shown in Figure 1. In unfolding conditions, the CD signal decreased to its final value within the mixing time. Reversibility of unfolding and refolding was checked by comparing the CD signal of the protein sample before and after unfolding and refolding.

Acknowledgments

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