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Cis proline mutants of ribonuclease A. I. Thermal stability

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Abstract

A chemically synthesized gene for ribonuclease A has been expressed in *Escherichia coli* using a T7 expression system (Studier, F.W., Rosenberg, A.H., Dunn, J.J., & Dubendorff, J.W., 1990, *Methods Enzymol. 185*, 60–89). The expressed protein, which contains an additional N-terminal methionine residue, has physical and catalytic properties close to those of bovine ribonuclease A. The expressed protein accumulates in inclusion bodies and has scrambled disulfide bonds; the native disulfide bonds are regenerated during purification. Site-directed mutations have been made at each of the two cis proline residues, 93 and 114, and a double mutant has been made. In contrast to results reported for replacement of trans proline residues, replacement of either cis proline is strongly destabilizing. Thermal unfolding experiments on four single mutants give $\Delta T_m \cong 10$ °C and $\Delta\Delta G^0$ (apparent) = 2–3 kcal/mol. The reason is that either the substituted amino acid goes in cis, and cis ⇔ trans isomerization after unfolding pulls the unfolding equilibrium toward the unfolded state, or else there is a conformational change, which by itself is destabilizing relative to the wild-type conformation, that allows the substituted amino acid to form a trans peptide bond.

Keywords: cis peptide bond; cis proline mutants; ribonuclease A; thermal stability

A problem of long-standing interest is whether or not the slow-folding forms of unfolded RNase A (Garel & Baldwin, 1973) are produced by cis ⇔ trans isomerization of the two cis proline residues, Pro 93 and Pro 114, a suggestion that follows logically from the proline isomerization model of Brandts et al. (1975). There is disagreement in the literature as to whether the amount of the trans isomer of either Pro 93 or Pro 114 is large enough to explain the proportion of the major slow-folding species of RNase A (Lin & Brandts, 1984; Adler & Scheraga, 1990). In the following paper (Schultz et al., 1992), we present experiments based on site-directed mutagenesis and folding kinetics that are aimed at answering this question.

It is necessary first to clone and express a gene for RNase A. This is made difficult by the toxicity of RNase A, which can digest cellular RNA (Richards & Wyckoff, 1971). We use a chemically synthesized gene for RNase A (the gift of Genex Corp.) and shut off its expression until induction by use of a T7 expression system (Studier et al., 1990) that includes T7 lysozyme, which reduces the basal level of T7 RNA polymerase in the uninduced state by binding it. Other ribonuclease expression systems are reviewed in the Discussion.

The two cis proline residues of RNase A (see Fig. 1A and Kinemage 1) both occur in type VI reverse turns, which by definition have a cis proline residue at position 3 (Creighton, 1984). The cis proline residues are exposed to solvent (see Fig. 1B and Richards & Wyckoff [1971]). Both turns have disulfide bonds nearby that may restrain their conformational freedom: the 40–95 S–S bond is close to the Pro 93 turn (Kinemage 3) and the 58–110 S–S bond is close to the Pro 114 turn (Kinemage 2).

Both Pro 93 and Pro 114 are highly conserved throughout evolution: Pro 93 is retained in the 40 known sequences of pancreatic ribonucleases, whereas Pro 114 is replaced by Leu in the capybara and is deleted in the kangaroo, wallaby, and turtle (Beintema et al., 1986). Substitution of a cis proline in evolution is frequently accompanied by an insertion or deletion (J. Richardson, pers. comm.). Although cis X-Pro peptide bonds occur commonly in folded proteins (6.5%, Stewart et al., 1990; MacArthur & Thornton, 1991), other cis peptide bonds are rare (0.05%, Stewart et al., 1990). Replacement of a

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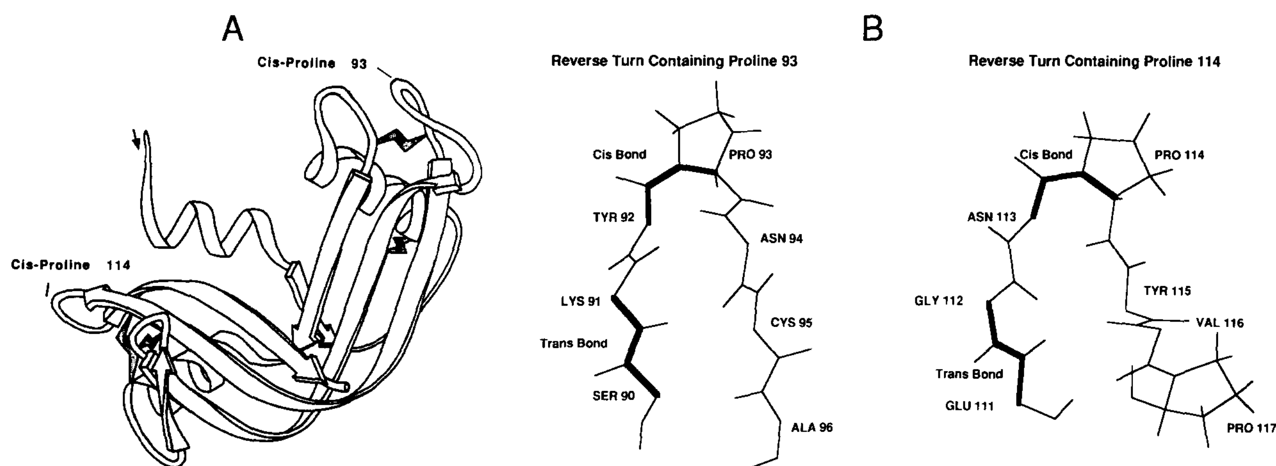

Fig. 1. **A:** A ribbon drawing by Jane Richardson of the structure of RNase A with the positions of the two cis proline residues, P93 and P114, identified. **B:** Main-chain structures of the two reverse turns containing P93 and P114, from the X-ray structure of RNase A (1986). The cis peptide bond is shown by a bold line and, for comparison, a trans peptide bond is also shown by a bold line.

cis proline residue necessarily produces either a cis non-proline peptide bond or else a change in backbone geometry (see Fig. 1B and Kinemage 4). Herzberg and Moult (1991) report that cis non-proline peptide bonds are found chiefly in regions of proteins that are connected with function. Thus, it is interesting to determine if a mutation at Pro 93 or Pro 114 allows the mutant protein to fold stably and form the correct four disulfide bonds of wild-type RNase A and, if so, whether the mutant protein is catalytically active; both Pro 93 and Pro 114 are remote from the active site.

If the amino acid that is substituted for a cis proline residue forms a cis peptide bond, this will automatically destabilize the folded protein relative to the unfolded form by at least 2 kcal/mol, which is a substantial energetic penalty. This happens because the trans:cis ratio of a non-proline peptide bond is about 100:1 (LaPlanche & Rogers, 1964; Drakenberg et al., 1972; Jorgensen & Gao, 1988), or even 1,000:1 (Ramachandran & Mitra, 1976). Unfolding and refolding can occur by the mechanism

$$N \rightarrow U_c \Leftrightarrow U_t \quad (1)$$

in which N is the folded conformation and U_c, U_t are unfolded forms with either a cis or trans peptide bond. Cis $\Leftrightarrow$ trans isomerization pulls the N $\Leftrightarrow$ U equilibrium to the right and contributes −2.7 kcal/mol to the ΔG^0 of unfolding if the cis:trans ratio is 1:100. Because the wild-type protein with a cis proline residue also undergoes cis $\Leftrightarrow$ trans isomerization after unfolding, a correction for this must be made. The cis:trans isomer ratio in heat-unfolded RNase A is about 1:2 for both P93 and P114, according to the NMR data of Adler and Scheraga (1990), and the contribution of cis $\Leftrightarrow$ trans isomerization to the ΔG^0 of unfolding is then −0.7 kcal/mol for each cis proline residue.

The replacements chosen for study here (P93A, P93S, P114G, P114A) were based on guesses as to which substitutions might allow stable folding.

Results

Properties of the expressed ribonuclease

The ribonuclease expressed in *Escherichia coli* is referred to as pseudo wild type because it contains an additional N-terminal methionine residue. Amino-terminal sequence analyses (data not shown) confirm that the five N-terminal residues have the expected sequence. Pseudo wild type comigrates with bovine RNase A in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Its specific enzymatic activity is close to that of bovine RNase A (see Materials and methods). The T_m (transition midpoint for thermal unfolding) of pseudo wild type is 1.5 °C higher than that of bovine RNase A (Table 1). This difference is not surprising in view of the results of Mitchinson and Baldwin (1986), who found that substitutions of the N-terminal residue affect the T_m of RNase S if the charge on the N-terminal residue, which can interact with the nearby positive pole of an α-helix dipole, is altered.

Because the engineered variants have specific enzymatic activities that are close to that of bovine RNase A, the variants are expected to have similar structures. The far-UV CD spectra of the variants are close to those of bovine RNase A and of pseudo wild type, as expected if their secondary structures are similar; Figure 3 compares the spectra of P114A and bovine RNase A. Marked differences from the UV absorption spectrum of bovine RNase A were found for certain variants (data not shown), but light scattering caused by aggregation was

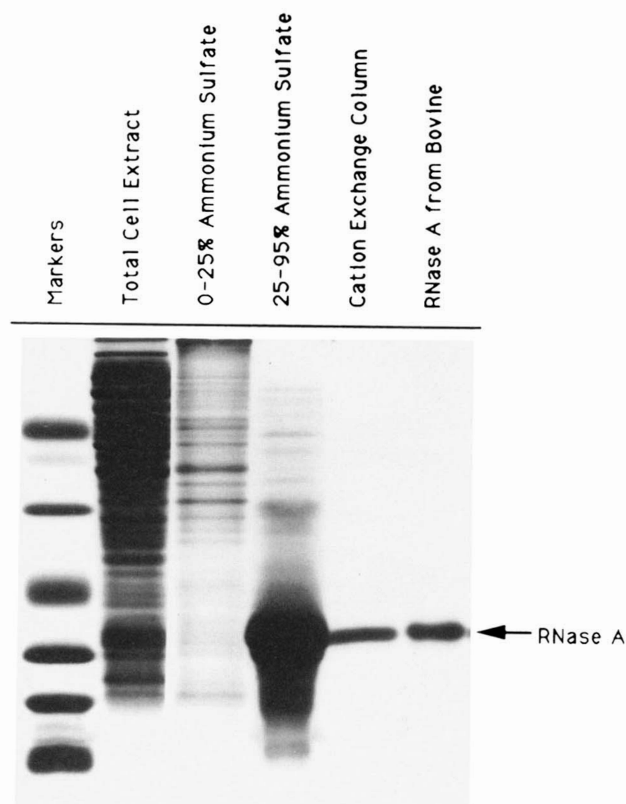

Fig. 2. SDS-PAGE gel showing stages in purification of pseudo wild-type RNase A. Lanes (from left to right) are as follows: (1) prestained molecular weight markers from Bethesda Research Laboratories; (2) total cell extract; (3) 0–25% ammonium sulfate pellet; (4) 25–95% ammonium sulfate pellet; (5) pooled fractions of pseudo wild type from a sulfopropyl Sephadex column; and (6) bovine RNase A from Sigma.

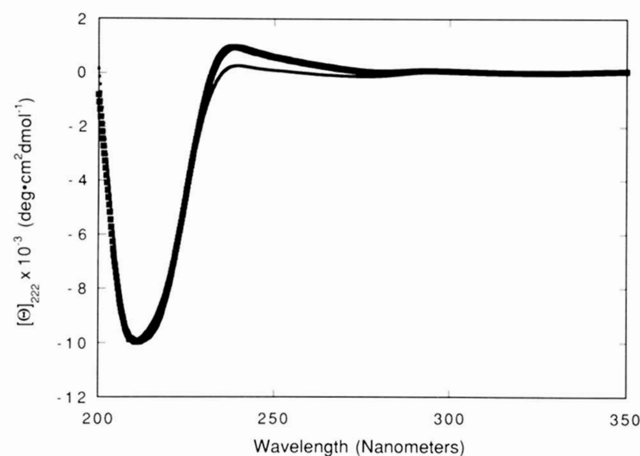

Fig. 3. CD spectra of bovine RNase A (thin line) and of the P114A mutant (bold line). Solvent: 0.1 M KF, pH 7.0, 2 °C, protein concentration about 10 μM, path length 1.0 cm. Data were collected by signal averaging for 1 s every 0.2 nm, and the spectra are averages of three or more repeated measurements. A 4-nm window was used to smooth the data. Concentrations were determined by absorbance (278 nm) in 6 M GdmCl (see Materials and methods).

Table 1. *Thermal stability of the mutant proteins*

Protein	T_m[a]	$-\Delta T_m$	$\Delta\Delta G^0_{app}$[b]
RNase A – bovine	54.5 (0.5)		
RNase A – *E. coli*[c]	56.0 (0.5)	0	
P93A	46.5 (0.5)	9.5	2.7 (0.4)
P93S	47.5 (0.5)	8.5	2.1 (0.4)
P114A	45.5 (0.5)	10.5	3.2 (0.4)
P114G	45.5 (0.5)	10.5	2.8 (0.4)
P93A, P114G	36.0 (1.0)	20	4.2 (1.0)

[a] Temperature midpoint of the thermal unfolding transition (measured by CD at pH 4.2, 0.1 M NaCl, 0.01 M Na acetate buffer). See Materials and methods for details. Standard errors are given in parentheses.

[b] The change in apparent free energy of unfolding, relative to the pseudo wild-type protein, at its T_m (329.2 °K). See Materials and methods for details. Standard errors are given in parentheses.

[c] The pseudo wild-type protein, containing an additional N-terminal methionine residue; expressed in *E. coli*.

sometimes a problem with the variant proteins at the higher concentrations needed for light absorption spectroscopy, and no serious study was made of these spectra.

Thermal stability of cis proline mutants

Transition curves for thermal unfolding were measured for all mutants. CD was adapted as the method of monitoring thermal unfolding, both because a low protein concentration (1–10 μM) can be used, which aids in obtaining reversible unfolding curves, and because the CD spectra of the variants closely resemble the spectrum of RNase A. Figure 4 shows the unfolding transition curve of the double mutant; comparison of the heating and cooling curves shows good reversibility. In Figure 5, the

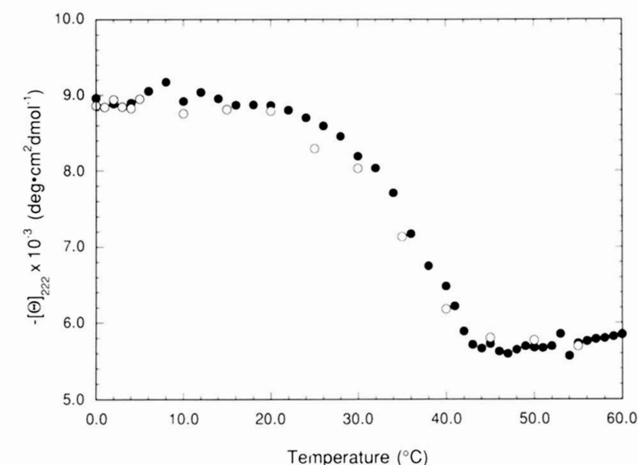

Fig. 4. Thermal unfolding transition of the double mutant P93A, P114G at pH 4.2. Heating curve, filled circles; cooling curve, open circles. See Materials and methods for details.

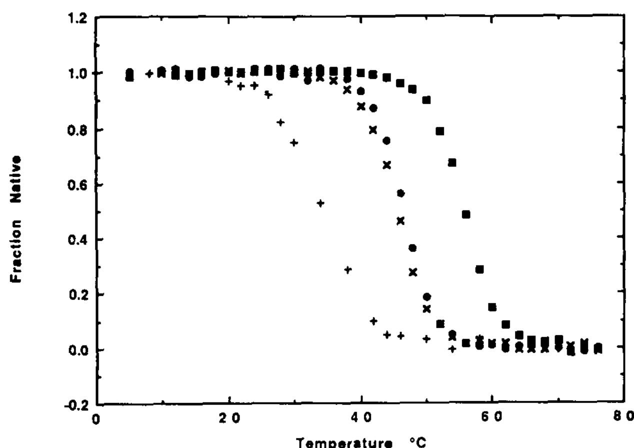

Fig. 5. Normalized unfolding transition curves, obtained by fitting linear baselines (see Materials and methods), for pseudo wild type (■), P93A (●), P114G (×), and the double mutant P93A, P114G (+).

unfolding transition curves have been normalized by fitting linear baselines, and the unfolding curves are compared for pseudo wild type, two single mutants, and the double mutant. Table 1 gives values of T_m and of the apparent ΔG^0 (standard free energy of unfolding) calculated for a two-state unfolding reaction.

In Table 1, the striking result is that each of the single mutants is destabilized to nearly the same extent, which is substantial ($\Delta T_m \sim 10$ °C), regardless of whether the mutation is at P93 or P114, and regardless of which amino acid (Ala, Gly, Ser) is substituted. Moreover, the double mutant shows a ΔT_m (20 °C) that is twice as large as those of the single mutants. Values for both T_m and ΔG^0 of unfolding are given in Table 1, but the T_m results are used for discussion because the ΔG^0 values are based on the assumption of two-state unfolding. Data in the following paper (Schultz et al., 1992) on the folding kinetics of the P93A and P93S mutants, show that the two-state unfolding model may not be correct for these mutants.

Discussion

Ribonuclease expression

Expression of RNase A presents a problem because of its toxicity and because failure to form the correct four disulfide bonds during synthesis results in a poorly folded or unfolded protein that is susceptible to protease degradation. Vasantha and Filpula (1989) have expressed in *Bacillus subtilis* the synthetic gene for bovine RNase A that we have studied here and have detected RNase catalytic activity in the culture supernatant. Before changing over to the T7 expression system described here, we began by expressing and purifying RNase A in this system (data not shown). McGeehan and Benner (1989) reported expression of RNase A in *E. coli* under control of the strong P_L promoter of phage lambda. RNase A has been expressed in yeast by secreting the expressed protein (Raines & Rutler, 1989). Angiogenin, which shows strong sequence homology to RNase A and has ribonuclease activity, has been expressed in *E. coli* under the control of the *trp* promoter (Denefle et al., 1987; Shapiro et al., 1988) and also has been expressed in baby hamster kidney cells (Kurachi et al., 1988). RNase T1 has been expressed in *E. coli* by linking the gene to the signal peptide of the *omp A* protein, so that active ribonuclease is secreted into the periplasmic space (Quaas et al., 1988). The expression system we use here (Studier et al., 1990) has been used recently to express a toxic protein, streptavidin (Sano & Cantor, 1990).

Decreased stability of cis proline mutants

When the proline residue is trans, replacement of proline by another amino acid typically produces a rather small change in stability and sometimes the replacement of the proline is stabilizing. This has been found for six proline mutations (either introduction or replacement of a proline residue) in human lysozyme (Herning et al., 1991) and also for five proline mutations in T4 lysozyme (Chen et al., 1992). There is a striking contrast between these results and the ones reported here for replacing either cis proline residue of RNase A.

The $\Delta\Delta G^0$ values found for the different single mutants studied here (2–3 kcal/mol, Table 1) are in the range estimated for introducing a cis non-proline peptide bond into the mutant protein. Data showing whether the bond is cis or trans are not yet available. Data for the kinetics of refolding (Schultz et al., 1992) suggest that the peptide bond may possibly be cis in the Pro 93 single mutants, and, if so, it is probably trans in the other mutants. If the substituted amino acid forms a cis bond, a sizable ΔG^0 (about 3 kcal/mol) is available to drive a conformational change that replaces the cis bond with a trans bond. Such a conformational change will often be strongly destabilizing relative to the wild-type conformation. Thus, replacement of a cis proline residue will often be destabilizing whether or not the substituted amino acid forms a cis peptide bond.

Another effect should contribute to the change in protein stability when either a cis or a trans proline residue is replaced: there should be an increase in the number of possible backbone conformations in the unfolded form, because of the limited number of backbone conformations of an X-Pro peptide bond (Schellman, 1955). This effect should stabilize the proline-containing protein by about 1.4 kcal/mol (Schellman, 1955; Matthews et al., 1987).* The effect is partly offset by the cis ⇔ trans isom-

* *Note added in proof*: Free-energy simulations indicate that this figure is an overestimate (Yun et al., 1991).

erization of the proline peptide bond in the unfolded state (see the introduction).

It is surprising that no substantial difference in stability is observed here between P93A and P93S or between P114G and P114A (Table 1). There is a difference in backbone flexibility between Ala and Gly, which should tend to make the Ala-containing protein more stable (Matthews et al., 1987). Other effects may, however, compensate, such as a difference in burial of nonpolar surface area.

Ihara and Ooi (1985) used energy minimization to investigate the structural consequences of replacing the cis proline residues of RNase A with trans conformers (see also the study of bovine trypsin inhibitor by Levitt [1981]). They predict that the structural differences between the cis and trans conformers are small and localized, both for Pro 93 and Pro 114.

Alternative folded conformations of a protein can coexist in which a proline residue is present either as the cis or trans conformer. This behavior has been observed both in nuclease (Evans et al., 1987, 1989) and in calbindin (Wendt et al., 1988; Skelton et al., 1990). In these instances, the Gibbs energy difference between the two folded conformations is small and a substituted amino acid is therefore likely to form a trans peptide bond. In nuclease, Pro 117 is cis in the folded conformation found when specific ligands are bound, and replacing Pro 117 by Gly gives a trans peptide bond (Hynes & Fox, unpubl.; cf. Evans et al., 1987).

Materials and methods

Gene for RNase A

A chemically synthesized gene for RNase A was the gift of Genex Corporation. It was contained in the plasmid pGX2213 (provided by Genex) and a signal peptide was attached to its N-terminus. To remove the signal peptide, the plasmid was digested with *Nde* I and *Sal* I, and the 1.4-kb DNA fragment containing most of the gene was isolated. A synthetic oligonucleotide containing *Nde* I overhangs and the coding sequence for the first 11 amino acids of RNase A plus N-terminal methionine were mixed with the 1.4-kb DNA fragment, and the mixture was ligated between the *Nde* I and *Sal* I sites of plasmid pT7-7. The correct orientation of the oligonucleotide in the construct was determined by dideoxy sequencing. The resulting plasmid is referred to as pRNA-1.

Bacterial strains and plasmids

Escherichia coli strains HMS174, BL21, TG1, and lysogens of these strains for the lambda derivative DE3 were provided by F.W. Studier and are described by Studier et al. (1990). DE3 contains the T7 RNA polymerase gene under the control of the *lac* UV5 promoter; the polymerase is inducible by isopropylthiogalactoside (IPTG). The plasmid pT7-7, which contains the translation initiation site for the T7 gene 10 protein, was provided by S. Tabor. The plasmids pLys S and pLys E, which contain the T7 lysozyme gene, were provided by F.W. Studier.

Materials

Enzymes for the construction of expression vectors were obtained from New England Biolabs and from Bethesda Research Labs. Oligonucleotides were synthesized by Operon. Bovine RNase A was obtained from Sigma (grade XIIA) and further purified by the procedure of Garel (1976). Reduced and oxidized glutathione, 2′,3′-cCMP (cyclic cytidine monophosphate), and IPTG were obtained from Sigma and Boehringer-Mannheim, respectively.

Constructs and growth

The strain BL21(DE3), carrying either the pLys E or pLys S plasmid was transformed with the plasmid pRNA-1. The procedure described by Sano and Cantor (1990) for expression of streptavidin was then used to express pseudo wild type RNase A and its mutants. The desired strain was grown overnight at 37 °C with shaking in 100 mL L-broth (Sambrook et al., 1989) containing 100 μg/mL carbenicilin and 25 μg/mL chloramphenicol. This culture was used to inoculate a 6-L flask containing 3 L L-broth, pH 7.5, supplemented with 0.4% glucose and antibiotics as described above. When A_{595} reached 0.5–0.7, IPTG was added to a final concentration of 0.4 mM. The cells were harvested 3 h later by centrifugation at 15,000 × *g* for 10 min, 4 °C. The cell pellets were then stored at −80 °C.

Inclusion body preparation

The frozen pellets were resuspended in 27 mL 50 mM Tris, pH 8.0, 1 mM EDTA; lysis occurred during incubation at room temperature for 30 min. The suspension was then sonicated or passed through a French press until the DNA was sheared and was centrifuged at 15,000 × *g* for 25 min, 4 °C. The pellets were resuspended in 7 mL cold 50 mM Tris, 300 mM NaCl, pH 8.0, with the use of a Dounce homogenizer. Pellets were collected by centrifugation, as described above, and the precipitate was dissolved with the aid of a Dounce homogenizer, in 10 mL 6 M GdmCl, 0.1 M Tris acetate, 1 mM EDTA, pH 8.5.

Reduction and oxidation

Reduced glutathione was added to the mixture to a final concentration of 0.1 M, and the pH was maintained at 8.5 with NaOH. The sample was left at room temperature for 2 h to allow complete reduction of all disulfide

bonds (Ahmed et al., 1975; Schaffer et al., 1975). The sample was then diluted 20-fold into 0.01 M Tris acetate, pH 8.5, and oxidized glutathione was added to a final concentration of 0.5 mM. The sample was then incubated for 12 h at room temperature. The disulfide bonds of RNase A were regenerated almost completely after 4 h oxidation, with no increase in yield after 12 h. Yields ranged from 0.5 to 2.0 mg RNase A per liter of culture.

Purification

First, 25% ammonium sulfate was added at room temperature: insoluble material was removed by centrifugation at 15,000 × *g* for 25 min, 4 °C. Ribonuclease was precipitated from the supernatant by adding ammonium sulfate to 95% and pelleting as above. The pellet was resuspended in 10 mM Tris Cl, pH 8.0, and diluted until the conductivity was less than 3 mMhos. The sample was loaded onto a sulfopropyl Sephadex column that was pre-equilibrated in the same buffer. The column was then washed with 10 mM Tris Cl, pH 8.0, adjusted to 3.5 mMho with NaCl. A 400-mL NaCl gradient, 0-300 mM NaCl, was applied; ribonuclease elutes around 200 mM NaCl. The peak fractions were pooled and lyophilized. The protein was then dissolved in water and centrifuged in a Centricon concentrator to remove the buffer and salt. All preparations were more than 95% pure as determined by SDS-PAGE.

Characterization

The concentrations of bovine RNase A and of pseudo wild type were determined by absorbance, using ϵ_{278} = 9,800 M^{-1} cm^{-1} (Sela & Anfinsen, 1957). The concentrations of the mutant proteins were determined either by amino acid analysis or by tyrosine absorbance at 278 nm in 6 M GdmCl, 0.05 M sodium phosphate, pH 6.5, using a molar absorbance based on the number of tyrosine residues (Edelhoch, 1967). Enzymatic activities were assayed with the substrate 2′,3′-cCMP (0.3 mg/mL) in 0.05 M Na cacodylate, pH 7.0, at 18 °C (Crook et al., 1960).

Mutagenesis

Pro 93 was substituted with Ala or Ser using the method of Kunkel et al. (1987); Pro 114 mutations were constructed by cassette mutagenesis with a degenerate oligonucleotide. Mutant sequences were confirmed by double-strand DNA sequencing.

Thermal unfolding

CD measurements were made with an Aviv 60DS spectropolarimeter using a cell with a 1.0-cm path length, protein concentration about 1-10 μM, and solvent 0.1 M NaCl, 0.01 M Na acetate buffer, pH 4.2. The ellipticity at 222 nm was measured at 2-4° intervals, after allowing 500 s for equilibration. Reversibility was tested by comparing the heating and cooling curves.

Linear baselines were fitted above and below the transition zone by the procedure of Santoro and Bolen (1988), and the two-state model (N ⇔ U, N = native, U = unfolded) was used to obtain apparent equilibrium constants at each temperature. The value of ΔG^0 (apparent) was calculated for each mutant protein at the T_m of pseudo wild type (329.2 °K) by the equation of Becktel and Schellman (1987).

$$\Delta G^0(T) = \Delta H_m(1 - T/T_m) - \Delta Cp[(T_m - T) + T\ln(T/T_m)]. \quad (2)$$

The value of ΔCp was taken as 2.2 $kcal \cdot mol^{-1} \cdot deg^{-1}$ (Pace & Laurents, 1989) and the value of ΔH_m, the enthalpy of unfolding at 329 °K, was found by fitting the transition curve to Equation 2.

Acknowledgments

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