FOR THE RECORD

Solution NMR evidence for a cis Tyr-Ala peptide group in the structure of [Pro93Ala] bovine pancreatic ribonuclease A

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Abstract: Proline peptide group isomerization can result in kinetic barriers in protein folding. In particular, the cis proline peptide conformation at Tyr92–Pro93 of bovine pancreatic ribonuclease A (RNase A) has been proposed to be crucial for chain folding initiation. Mutation of this proline-93 to alanine results in an RNase A molecule, P93A, that exhibits unfolding/refolding kinetics consistent with a cis Tyr92–Ala93 peptide group conformation in the folded structure (Dodge RW, Scheraga HA, 1996, *Biochemistry* 35:1548–1559). Here, we describe the analysis of backbone proton resonance assignments for P93A together with nuclear Overhauser effect data that provide spectroscopic evidence for a type VI β -bend conformation with a cis Tyr92–Ala93 peptide group in the folded structure. This is in contrast to the reported X-ray crystal structure of [Pro93Gly]-RNase A (Schultz LW, Hargraves SR, Klink TA, Raines RT, 1998, *Protein Sci* 7:1620–1625), in which Tyr92–Gly93 forms a type-II β -bend with a trans peptide group conformation. While a glycine residue at position 93 accommodates a type-II bend (with a positive value of ϕ_{93}), RNase A molecules with either proline or alanine residues at this position appear to require a cis peptide group with a type-VI β -bend for proper folding. These results support the view that a cis Pro93 conformation is crucial for proper folding of wild-type RNase A.

Keywords: cis peptide groups; protein folding mechanisms; protein NMR

The protein folding problem is one of the most important challenges in the field of molecular biophysics, and bovine pancreatic ribonuclease A (RNase A) is one of the most extensively charac-

terized model systems in the protein folding literature (Neira & Rico, 1997; Raines, 1998). Both kinetic and thermodynamic studies have provided information about the nature of folding intermediates and the roles of specific interactions in determining rate-limiting steps on several parallel folding pathways. Early kinetic studies of the folding mechanism of disulfide-intact RNase A demonstrated that its refolding occurs along parallel pathways corresponding to a heterogeneity of the unfolded state (Garel & Baldwin, 1973). Subsequent studies have identified this heterogeneity with specific cis/trans isomeric states of the four prolines in the ensemble of unfolded species (Brandts et al., 1975; Schmid & Baldwin, 1978; Cook et al., 1979; Houry et al., 1994; Nall, 1994; Dodge & Scheraga, 1996; Juminaga et al., 1997, 1998).

In the native folded structure of RNase A, two of the X-Pro peptide groups (Lys41–Pro42 and Val116–Pro117) have trans conformations, while the other two (Tyr92–Pro93 and Asn113–Pro114) have cis conformations (Wlodawer et al., 1988). Upon unfolding, however, the ensemble of disulfide-intact RNase A molecules includes an equilibrium distribution of cis and trans conformers at each of its four X-Pro peptide groups (Brandts et al., 1975; Lin & Brandts, 1983a, 1983b). The cis/trans conformations of three of these X-Pro peptide groups (Pro93, Pro114, and Pro117) have been shown to affect the rates of conformational folding (Dodge & Scheraga, 1996), giving rise to eight isomeric states. These eight forms fold at different rates, resulting in multiphasic folding kinetics. One of these folding phases is rate-limited by proline isomerization, but the others are not; i.e., nonnative proline isomers slow protein folding but generally do not block it (Cook et al., 1979; Houry & Scheraga, 1996). In particular, the isomeric state of Pro93 is critical for conformational folding; a nonnative trans isomer of Pro93 retards folding almost 1,000-fold (Dodge & Scheraga, 1996; Juminaga et al., 1998).

The role of proline isomerization has been elucidated by extensive biophysical studies of folding kinetics and the effects of proline mutations on these kinetics. Single- and double-jump studies of protein unfolding/refolding kinetics (Cook et al., 1979; Lin & Brandts, 1983a, 1983b; Houry et al., 1994; Dodge & Scheraga, 1996; Houry & Scheraga, 1996; Juminaga et al., 1997, 1998) provide data on the interconversion between the transient, initially-

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Abbreviations: 2QF-COSY, two-quantum filtered proton–proton correlated spectroscopy; 2D, two-dimensional; HPLC, high-performance liquid chromatography; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; RNase A, bovine pancreatic ribonuclease A; P93A and P93G, single-site mutants of RNase A containing Ala and Gly, respectively, at position 93; TOCSY, total proton–proton correlation spectroscopy.

generated unfolded state of RNase A (unfolded species U_{uf} with native X-Pro peptide bond conformations) and the ensemble of unfolded states with one or more nonnative X-Pro peptide group conformations (unfolded species U_f , U_m , U_s^I , and U_s^{II}). Significantly, double-jump unfolding/refolding kinetic studies of the single-site mutant Pro93Ala RNase A (P93A) (Dodge & Scheraga, 1996) reveal a U_{uf} species similar to that observed for wild-type RNase A that can refold rapidly to the native state or, if left under unfolding conditions, convert into a significant population of slower-folding species. Dodge and Scheraga (1996) have interpreted kinetic data on the refolding of U_{uf} in P93A as indicating that the Tyr92–Ala93 peptide group in the folded mutant, and in the corresponding U_{uf} species, is in the cis conformation, as is the Tyr92–Pro93 peptide group in folded wild-type RNase A and its corresponding U_{uf} species. This interpretation of kinetic data on the refolding of P93A is consistent with studies of conformational preferences of peptide fragments of RNase A, which demonstrate that a cis peptide bond conformation in the Tyr92–Pro93–Asn94 sequence is required to form low-energy β -bend conformations (Oka et al., 1984). The resulting chain reversal [a type-VI β -bend (Lewis et al., 1973) that results from a cis Pro peptide group conformation] is thought to be essential for chain-folding initiation on some pathways in the folding process (Montelione et al., 1984; Oka et al., 1984; Montelione & Scheraga, 1989).

To validate the hypothesis that the folded structure of P93A contains a nonproline cis Tyr92–Ala93 peptide group conformation, the X-ray crystal structure of P93A was determined at 2.3 Å resolution by difference Fourier methods (Pearson et al., 1998). Crystallographic B factors are generally higher in this crystal structure of P93A than in the wild-type structure. In particular, residues 88 through 94 of the P93A structure are poorly resolved in the electron density. As the polypeptide chain could be modeled through this density with either a cis or trans Tyr92–Ala93 peptide group conformation (Pearson et al., 1998), the crystallographic data do support the identification of a cis peptide group at this site in P93A, but not unambiguously. In this paper, we describe solution NMR data for P93A that demonstrate unambiguously that the Tyr92–Ala93 peptide group is indeed cis in this native structure.

Results and discussion: Homonuclear NOESY spectra can provide unambiguous data for distinguishing cis from trans peptide groups (Wüthrich et al., 1984). Specifically, cis peptide group conformations of secondary or tertiary amides result in H_i^α – H_{i+1}^α interproton distances ranging from about 2.0–3.8 Å (depending on the values of the dihedral angles ψ_i and ϕ_{i+1}) while, for trans peptide groups, the corresponding H_i^α – H_{i+1}^α interproton distances are 4.2–4.8 Å (Wüthrich et al., 1984). In the absence of significant spin diffusion effects, observation of a strong sequential H_i^α – H_{i+1}^α NOE provides direct evidence for a cis peptide group conformation. For X-Pro peptide groups, sequential H_i^α – H_{i+1}^δ NOEs are also diagnostic of trans peptide groups (Wüthrich et al., 1984).

An essentially complete set of ^1H , ^{13}C , and ^{15}N backbone resonance assignments, based on three-dimensional (3D) triple-resonance NMR spectra, has been reported for wild-type RNase A at pH 4.6 and a temperature of 20 °C (Shimotakahara et al., 1997). These resonance assignments are in generally good agreement with ^1H resonance assignments determined at pH 3.2 and 30 °C (Robertson et al., 1989) and at pH 4.0 and 35 °C (Rico et al., 1989) using 2D homonuclear NMR methods. Resonance assignments for wild-type RNase A determined at 20 °C (Shimotakahara et al.,

1997) were further validated by following the sequential backbone connectivities in a 2D homonuclear NOESY spectrum recorded at 20 °C on a sample of wild-type RNase A prepared at pH 4.6 ± 0.1 , using a NOESY mixing time of $\tau_m = 80$ ms. These ^1H resonance assignments for wild-type RNase A were then used as the basis for analysis of 2D homonuclear spectra recorded at 20 °C on a sample of P93A, also prepared at pH 4.6 ± 0.1 . The spectra collected for the P93A mutant sample included 2D NOESY (using $\tau_m = 80$ ms), 2D TOCSY (using isotropic mixing times of 20 and 40 ms), and 2D 2QF-COSY. A summary of the sequential H_i^N – H_{i+1}^N and H_i^α – H_{i+1}^α NOEs used to establish the backbone H^N and H^α resonance assignments for P93A is presented in Figure 1. Some side-chain ^1H resonance assignments were also determined from these data for P93A. These proton resonance assignments for P93A have been deposited in the BioMagResBank.

Also shown in the schematic diagram of Figure 1 is a summary of a comparison between the H^N and H^α chemical shifts of P93A and wild-type RNase A. These proton chemical shifts are essentially identical throughout the molecule, except in the vicinity of the Pro93Ala substitution. The close similarity of backbone proton chemical shifts of P93A and wild-type RNase A demonstrate that the backbone conformation and overall structures of these two molecules are identical, except for some local structural perturbations in the Tyr92–Ala93 polypeptide segment. A small chemical shift difference is also observed for the H^N resonance of residue Cys40, which forms a disulfide bond with Cys95 and is nearby, in 3D space, the site of the P93A mutation. Visual inspection of backbone proton linewidths did not indicate unusual conformational exchange or other dynamic differences between the wild-type and mutant proteins, and there is no indication of significant disorder in the polypeptide segment of P93A containing residues Tyr92 and Ala93, as was suggested by the crystallographic analysis (Pearson et al., 1998). However, a more extensive dynamic study would be required to reliably characterize any effects that the Pro93Ala mutation might have on the dynamics of RNase A.

With resonance assignments for both P93A and wild-type RNase A in hand, the 80 ms NOESY spectra were next examined to identify cross peaks between the H^α resonance of Tyr92 and the H^α resonances of Pro93 or Ala93. The relevant regions of the NOESY spectra of the wild-type and mutant proteins are shown superimposed in Figure 2. The $H^\alpha(\text{Y92})$ – $H^\alpha(\text{P93})$ cross peak resulting from the cis Tyr92–Pro93 peptide group is easily identified in the spectrum of the wild-type protein (in blue). The corresponding $H^\alpha(\text{Y92})$ – $H^\alpha(\text{A93})$ cross peak resulting from the cis Tyr92–Ala93 peptide bond is also easily identified in the spectrum of P93A (in red). These sequential $H^\alpha(92)$ – $H^\alpha(93)$ NOESY cross peaks of wild-type and P93A RNase A have similar intensities, suggesting similar values of the dihedral angles ψ_{92} and ϕ_{93} in the mutant and wild-type proteins. The turn involving residues 91–92–93–94 was confirmed by identification of (partially overlapped) $H^\alpha(\text{Y92})$ – $H^\beta(\text{N94})$ and strong $H^\delta(\text{Y92})$ – $H^\alpha(\text{A93})$ NOEs consistent with a native-like chain reversal.

In Figure 2 there is a second NOESY cross peak downfield of the one assigned to the crucial Tyr92 H^α –Ala93 H^α interaction. A peak at this position is also observed in the TOCSY spectrum of the mutant, indicating that it is an intraresidue cross peak (Fig. 3). This peak could not be assigned unambiguously from our data; several assignments are possible. However, it is resolved from the key NOESY cross peak of interest (i.e., the Tyr92 H^α –Ala93 H^α NOESY cross peak labeled in Figs. 2, 3). Superimposing the NOESY and TOCSY data (Fig. 3), it is clear that this unassigned peak, as

Fig. 1. Survey of sequential $H_i^N-H_{i+1}^N$ [$NN(i,i+1)$] and $H_i^N-H_{i+1}^\alpha$ [$\alpha N(i,i+1)$] NOE data for P93A RNase A obtained at pH 4.6 and a temperature of 20 °C from a NOESY spectrum recorded with a mixing time of $\tau_m = 80$ ms. Also shown are differences in chemical shift $\Delta\delta = \delta_{P93A} - \delta_{wt-RNase A}$ for the backbone H^N and H^α resonances.

well as the Cys 26 $H^\alpha-H^\beta$ cross peak assigned in the TOCSY spectrum, are distinct from the NOESY peak assigned to the Tyr92 H^α -Ala93 H^α interaction. The assigned H^α (Y92)- H^α (A93) NOESY cross peak (3.90 ppm, 3.12 ppm) also cannot be attributed to an intrasidue Tyr92-Tyr92 H^β interaction, as superposition of the NOESY and TOCSY spectra (Fig. 3) reveals that there is no intrasidue TOCSY cross peak corresponding to this NOESY cross peak. Moreover, the assigned resonance frequencies of the two Tyr92 H^β resonances are 3.48 and 2.74 ppm, respectively.

The 80 ms NOESY mixing time used in this study is sufficiently short to avoid significant spin-diffusion effects. Except for the Tyr92-Ala93 peptide group of P93A no other sequential $H_i^N-H_{i+1}^\alpha$ NOEs were observed for the many other secondary peptide bonds of the wild-type and mutant proteins. For P93A, NOESY cross peaks are also observed at positions expected for $H^{\beta 2}$ (Y92)- H^α (A93) and $H^{\beta 3}$ (Y92)- H^α (A93) NOESY interactions of a cis peptide group, although these NOESY cross-peak assignments are not absolutely unambiguous owing to degeneracies of these H^β resonance frequencies with those of other P93A protons. Overall, the NOESY data provide an unambiguous determination of the Tyr92-Xxx93 peptide group conformations in P93A and wild-type RNase A, both of which are cis.

These NMR data provide spectroscopic and structural confirmation of the hypothesis, based on kinetic folding studies, that the

Tyr92-Ala93 peptide group of P93A is cis in the folded structure (Dodge & Scheraga, 1996). In RNase A, it appears that a cis peptide group at Tyr92-Pro93 is essential for forming a type-VI β -bend that can support folding of the protein molecule. This was in fact exactly the conclusion of extensive experimental and computational analysis of the conformational properties of this sequence (Tyr-Pro-Asn) in polypeptide fragments of RNase A with cis and trans Tyr-Pro peptide groups (Montelione et al., 1984; Oka et al., 1984; Montelione & Scheraga, 1989). In P93A, it appears that a trans Tyr92-Ala93 peptide group does not accommodate the type of bend formation that is required for folding (Oka et al., 1984), while the generally less populated cis conformational ensemble can form a native-like type-VI β -bend [i.e., a chain reversal resulting from a cis peptide group conformation (Lewis et al., 1973)] that allows the mutant molecule to fold to a native structure that is essentially identical to wild-type RNase A.

Although they are energetically unfavorable in small peptides (Zimmerman & Scheraga, 1976, 1977; Zimmerman et al., 1977), cis peptide groups do occur in the database of protein structures. Analysis of the 3D structures of a nonredundant set of 1,106 proteins (Hobohm & Sander, 1994), comprising a total of 238,097 residues, reveals 36 protein structures with 67 cis peptide groups (0.028%). Of these 67 cis peptide groups, 19 (28%) involve non-prolyl peptide groups. Mutagenesis studies have also indicated a

Fig. 2. Expanded regions from 2D NOESY spectra of wild-type RNase A (blue contours) and P93A mutant RNase A (red contours). Both NOESY spectra were recorded at 20 °C using samples prepared at $\text{pH } 4.6 \pm 0.1$ and ~ 2.5 mM protein concentration, with a mixing time $\tau_m = 80$ ms. Key $\text{H}_i^\alpha\text{--H}_{i+1}^\alpha$ NOEs of cis Tyr92–Pro93 (in wild-type RNase A) and cis Tyr92–Ala93 (in P93A) peptide groups are indicated.

crucial role for cis peptide groups in the folding of other proteins. For example, nonprolyl cis peptide groups have also been reported in structures of cis-Pro mutants Pro202Ala human carbonic anhydrase II (Tweedy et al., 1993), Pro39Ala ribonuclease T1 (Mayr et al., 1994), and Pro195Ala *Escherichia coli* aspartate aminotransferase (Birolo et al., 1999). In these specific sites, as for Pro92 in RNase A, a cis peptide group conformation is required for folding even if the corresponding proline residue is mutated to alanine.

In a high-resolution crystallographic study (Schultz et al., 1998) of a related mutant, Pro93Gly RNase A (P93G), difference Fourier analysis of data at 1.7 Å resolution reveals a type-II β -bend at Tyr92–Gly93, with a peptide group that is unambiguously in the trans conformation. Unfortunately, double-jump kinetic folding data are not available for P93G. The NMR data for P93A clearly exclude a type-II β -bend at Tyr92–Ala93, as the corresponding $\text{H}^\alpha\text{--H}^\alpha$ distance would be greater than 4 Å (Wüthrich et al., 1984) and would not result in the observed strong NOESY cross peak. Although minor conformers were not identified in the analysis of these NMR data for P93A, a small equilibrium population of molecules with trans Tyr92–Ala93 peptide groups cannot be absolutely excluded.

A type-II β -bend requires that the residue at position 2 (in this case, residue position 93) adopt a conformation with a positive value of the backbone dihedral angle ϕ (Venkatachalam, 1968). While this is energetically unfavorable for both Pro and Ala (Lewis et al., 1973; Zimmerman et al., 1977), it is energetically accessible

for Gly residues, which in fact occur frequently in the second position of type II β -bends (Wilmot & Thornton, 1988). For these reasons, local interactions in the Tyr–Gly–Asn sequence of unfolded P93G are expected to allow formation of chain reversals (e.g., type-II β -bends) with trans peptide bonds in the initial stages of folding. These bend conformations are facilitated by the presence of a Gly residue and would be energetically costly for wild-type RNase A or P93A. As has been suggested by Schultz et al. (1998), these conformational preferences of the Tyr–Gly–Asn sequence apparently allow folding to a native P93G structure with the type-II β -bend observed in the crystal structure without requiring the nonproline cis peptide group conformation observed in P93A.

Materials and methods: Protein purification: The gene for [P93A]-RNase A, cloned in the pET22b(+) production vector (Novagen, Madison, Wisconsin), was expressed in *E. coli* strain BL21(DE3) (Novagen) as described previously (Dodge & Scheraga, 1996). Inclusion bodies were isolated by centrifugation, dissolved in 7 M GdnHCl containing 25 mM Tris-HCl, 5 mM EDTA and 0.3 M Na_2SO_3 at pH 8.0, and then S-sulfonated according to procedures described elsewhere (Thannhauser & Scheraga, 1985). The S-sulfonated protein was next purified by dialysis against 50 mM formic acid containing 3 M urea at pH 4.0 at 4 °C, and then buffer-exchanged into 0.1 M Tris-HCl containing 3 mM EDTA at pH 8.2

Fig. 3. Expanded regions from superimposed 2D NOESY (red contours) and TOCSY (green contours) spectra of P93A mutant RNase A recorded with mixing times of 80 and 40 ms, respectively. The TOCSY cross peak just downfield of the $H_{Y92}^{\alpha}-H_{A93}^{\alpha}$ cross peak in the ω_1 dimension is assigned to a Cys26 $H^{\alpha}-H^{\beta}$ intraresidue cross peak. The superimposed TOCSY/NOESY cross peak just downfield of the $H_{Y92}^{\alpha}-H_{A93}^{\alpha}$ cross peak in the ω_2 dimension is another intraresidue cross peak that could not be assigned unambiguously from the available data.

(regeneration buffer) using a pre-equilibrated 45 cm $\times$ 2.5 cm Sepharose G-25M (Pharmacia, Uppsala, Sweden) column. For regeneration, the eluted S-sulfonated protein was diluted to a protein concentration of 0.2–0.4 mg/mL in regeneration buffer, and oxidized and reduced glutathione were added to concentrations of 1 and 2 mM, respectively. Disulfide regeneration was carried out at 4 °C for 48 h and then stopped by lowering the pH to 5 with glacial acetic acid. The solution was next concentrated using an Amicon device (YM-3 membrane) and purified using a preparative Rainin Hydropore cation-exchange column with a linear gradient of 0–15% NaCl and flow rate of 8 mL/min. The purified protein was concentrated using an Amicon concentrator (YM-3 membrane) and then buffer exchanged into 100 mM acetic acid by dialysis. HCl was then added to a final concentration of 2–3 mM, and the solution was frozen and lyophilized. The addition of HCl prior to the lyophilization suppresses the large acetate resonance in the subsequent NMR measurements.

NMR spectroscopy: Samples of P93A and wild-type RNase were prepared for NMR experiments at protein concentrations of

~2.5 mM in 95% H_2O , 5% D_2O solutions containing 0.5 mM NaN_3 . These samples were adjusted to pH 4.6 ± 0.1 using dilute solutions of HCl and NaOH, and then transferred into 5 mm Shigemi susceptibility-matched NMR tubes. All NMR data were collected at 20 °C on a Varian Unity 500 NMR spectrometer. The programs VNMR (Varian Associates, Palo Alto, California), NMRCompass (Molecular Simulations, Inc., Burlington, Massachusetts), and SPARKY (T. James & T. Goddard, unpubl.) were used for data processing and analysis. Proton chemical shifts were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonic acid.

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