Protein Science (1998), 7:1255–1258. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Crystal structures of two mutants that have implications for the folding of bovine pancreatic ribonuclease A

MATTHEW A. PEARSON,[1] P. ANDREW KARPLUS,[1] ROBERT W. DODGE,[2] JOHN H. LAITY,[2] AND HAROLD A. SCHERAGA[2]

[1]Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853
[2]Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301

(RECEIVED November 3, 1997; ACCEPTED February 20, 1998)

Abstract: The Tyr92–Pro93 peptide group of bovine pancreatic ribonuclease A (RNase A) exists in the *cis* conformation in the native state. From unfolding/refolding kinetic studies of the disulfide-intact wild-type protein and of a variant in which Pro93 had been replaced by Ala, it had been suggested that the Tyr92–Ala93 peptide group also exists in the *cis* conformation in the native state. Here, we report the crystal structure of the P93A variant. Although there is disorder in the region of residues 92 and 93, the best structural model contains a *cis* peptide at this position, lending support to the results of the kinetics experiments. We also report the crystal structure of the C[40, 95]A variant, which is an analog of the major rate-determining three-disulfide intermediate in the oxidative folding of RNase A, missing the 40–95 disulfide bond. As had been detected by NMR spectroscopy, the crystal structure of this analog shows disorder in the region surrounding the missing disulfide. However, the global chain fold of the remainder of the protein, including the disulfide bond between Cys65 and Cys72, appears to be unaffected by the mutation.

Keywords: *cis-trans* isomerization; crystal structures of RNase A variants; disorder; X-Pro and X-Ala peptide groups

Kinetic and NMR studies of the folding of bovine pancreatic ribonuclease A (RNase A) are being carried out to identify the interatomic interactions that lead to the folded protein structure. RNase A contains four disulfide bonds and four proline residues (two *cis* and two *trans*) (Wlodawer et al., 1988), and kinetic folding studies have been carried out both with the disulfide bonds intact (Garel & Baldwin, 1973; Houry et al., 1994; Dodge & Scheraga, 1996) and with the disulfide bonds first reduced and then oxidized (Rothwarf et al., 1998).

Mutants of RNase A have been examined to identify the roles of key residues in the folding mechanism. In guanidine hydrochloride-induced unfolding of disulfide-intact RNase A, the peptide groups preceding proline isomerize to an ensemble of species containing mixtures of *cis* and *trans* groups at each of these sites (Garel & Baldwin, 1973). The first unfolded species to form is U_{vf} (Houry et al., 1994), a very fast refolding species in which these four X-Pro groups retain their native *cis/trans* conformation. The concentration of U_{vf} decreases rapidly as the X-Pro groups isomerize to the equilibrium isomeric-state populations. In a stopped-flow refolding kinetic experiment, U_{vf} folds very rapidly to the native structure without requiring peptide-group isomerization (Houry et al., 1994) which, as shown by Brandts et al. (1975), would otherwise slow the refolding process.

The distribution of *cis* and *trans* proline peptide groups in the unfolded state has been deduced from stopped-flow unfolding/refolding kinetic studies of the wild-type and of proline-to-alanine and tyrosine-to-phenylalanine mutants of RNase A (Dodge & Scheraga, 1996; Houry & Scheraga, 1996; Juminaga et al., 1997). In these studies, the U_{vf} species of the proline-93-to-alanine (P93A) mutant of RNase A exhibited very different kinetic properties than the U_{vf} species of wild-type RNase A. The U_{vf} species of P93A converts to other unfolded species (U_m and U_s) at a rate that is 20 times greater than that at which the U_{vf} species converts to other unfolded species in wild-type RNase A (Table 7 of Dodge & Scheraga, 1996). U_{vf} is the unfolded species that has peptide groups in their native isomeric states, and therefore, the rapid rate of disappearance of U_{vf} in P93A suggested that the isomeric states of peptide groups occurring in the native state were energetically unfavorable in the unfolded state. Because a *cis* Tyr92–Ala93 peptide bond would be energetically very unfavorable compared to a *trans* Tyr92–Ala93 peptide bond in the *unfolded* protein (Zimmerman & Scheraga, 1976), the unfolding kinetics of the U_{vf} species in P93A RNase A were postulated to be due to a *cis* Tyr92–Ala93 peptide group in the native state which, upon unfolding, rapidly converts to a *trans* peptide group. Thus, it is possible that, in the folded form of the P93A variant, long-range interactions overcome the free energy deficit caused by a *cis* peptide group present before a non-proline residue.

In the oxidative folding of RNase A from the reduced form, two rate-determining three-disulfide intermediates have been identified along the folding pathway, des-(65–72) and des-(40–95), missing

Reprint requests to: H.A. Scheraga, Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301; e-mail: has5@cornell.edu.

the 65–72 and 40–95 disulfide bonds, respectively (Rothwarf et al., 1998). These same two intermediates were observed in the rate-determining steps of the reductive unfolding of RNase A (Li et al., 1995). To obtain structural information about these intermediates, two mutants were prepared as analogs. In one variant, Cys65 and Cys72 were each replaced by serine, and in the other Cys40 and Cys95 were each replaced by alanine. NMR structural studies of both mutants (Shimotakahara et al., 1997; Laity et al., 1997) indicated that the overall fold of these analogs resembles the native conformation of wild-type RNase A. However, significant structural perturbations were observed in the respective regions of the missing disulfide bonds, as well as more subtle changes in other regions far from the sites of mutation.

This report describes the X-ray crystal structures of the native (folded) forms of the P93A and C[40, 95]A variants in order to examine the suggestion from the kinetic experiments that the Tyr92–Ala93 peptide bond is *cis* in the folded P93A protein, and the conclusion from the NMR experiments about disorder in the region of the missing disulfide bond in the C[40, 95]A variant.

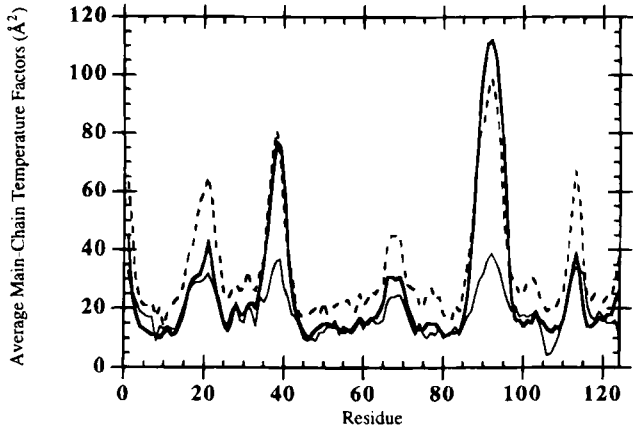

Fig. 1. Disorder in the P93A and (C40A, C95A) variants. The average main-chain temperature factors (Å^2) are plotted for all residues in the structures of the semisynthetic RNase A (Martin et al., 1987) (thin solid line), the P93A variant (dashed line), and the C[40, 95]A variant (thick solid line).

Results and discussion: The P93A and C[40, 95]A ribonuclease variants were crystallized in the space group observed for crystals of a semisynthetic ribonuclease (Martin et al., 1987). Because the structure of this semisynthetic enzyme (PDB entry 1SRN) is nearly identical to that of the wild-type protein (Wlodawer et al., 1988; PDB entry 9RSA), the structures of the P93A and C[40, 95]A variants were determined by difference Fourier methods, at resolutions of 2.3 and 1.6 Å, respectively, using the structure of the semisynthetic ribonuclease as the starting model. The models of the variant enzymes were then refined to *R*-factors of 21–22% (see Methods). Both of the refined structures are very similar to the structure of wild-type RNase A reported by Wlodawer et al. (1988); the root-mean-square deviation between C^α positions is 0.38 Å for the P93A variant and the wild-type enzyme, and is 0.44 Å for the C[40, 95]A and wild-type proteins. The major differences between the structures of both variants and the wild-type enzyme are in residues 37–40 and 88–95, near the sites of mutation.

In the P93A variant, there are clear increases in disorder, as shown by comparing the refined main-chain temperature factors of the variant and the semisynthetic ribonuclease (Fig. 1). The temperature factors of the semisynthetic protein are used for this comparison to ensure that the observed differences are not due to a change in crystal packing interactions. The temperature factors of the P93A variant are generally higher than those in the semisynthetic enzyme, which suggests lattice disorder in the crystal. This disorder may be due to the transfer of the crystal to a new mother liquor (see Methods), and appears to contribute to the lower resolution of the variant structure (2.3 vs. 1.8 Å for the semisynthetic structure), but there are also two regions of the variant where the increase is more pronounced. The loop containing Ala93 becomes disordered as shown by the average main-chain temperature factors for residues 89–94, which are 80–100 Å^2 in the variant and 30–40 Å^2 in the semisynthetic structure. The increase in disorder makes the electron density in this region difficult to interpret. The conformation of the peptide bond between residues 92 and 93 is not unambiguously defined; hence, alternative refinements were carried out with a *cis* or a *trans* peptide bond at this position (Fig. 2), yielding models with essentially the same *R*-factors. The *cis* conformation (Ala 93: $\phi = -104°$, $\psi = 30°$) closely resembled the wild-type conformation ($\phi = -92°$, $\psi = 14°$). Although the *trans* conformation appeared to fit the density reasonably well, it leads to torsion angles for Ala93 ($\phi = 113°$, $\psi = -26°$) that have never been observed for alanine residues in a database of high-resolution protein crystal structures (Karplus, 1996). By contrast, this database contains five examples of nonproline residues that are preceded by *cis* peptide bonds. Thus, the most plausible interpretation is that the peptide bond between Tyr92 and Ala93 is in the *cis* conformation. It is clear that the loop conformation present in the wild-type enzyme is destabilized by the mutation of Pro93 to alanine. The second region of the P93A variant that shows increased disorder is the loop containing residues 36–39, which have main-chain temperature factors of 55–80 Å^2 in the variant and 25–40 Å^2 in the semisynthetic protein. This is not surprising, considering that residues 36–40 of this loop pack against the disordered loop containing residues 92–93, and that the loops are linked by the disulfide between Cys40 and Cys95.

In the C[40, 95]A variant, the NMR data indicated that residues 34–45 and 83–101 have increased levels of disorder and that there was significant destabilization of the hydrophobic core (Laity et al., 1997). The crystal structure also shows disorder in these regions, as shown by the lack of clear electron density and an increase in the main-chain temperature factors to 50–80 Å^2 for residues 36–40 and 70–110 Å^2 for residues 90–95 (Fig. 1). The lack of electron density in the loop containing Pro93 again makes the conformation of the peptide bond between residues 92 and 93 somewhat ambiguous, although the *cis* conformation does fit the observed density. The crystal structure of the variant also shows that residues 87–89 clearly shift in position by 0.5–0.7 Å. There are no other regions of the crystal structure that show clear structural shifts or increased disorder. This indicates that the changes in chemical shift, J-coupling, and amide proton exchange that were observed in the NMR experiments for residues far from the sites of the mutation (Laity et al., 1997) are due to subtle changes in structure and/or conformational flexibility, associated with the decreased thermodynamic stability, that are not apparent in the average structure obtained crystallographically.

In conclusion, the crystal structures of P93A and C[40, 95]A ribonuclease corroborate the results of earlier kinetic and NMR experiments. In both variants, increased disorder is observed for

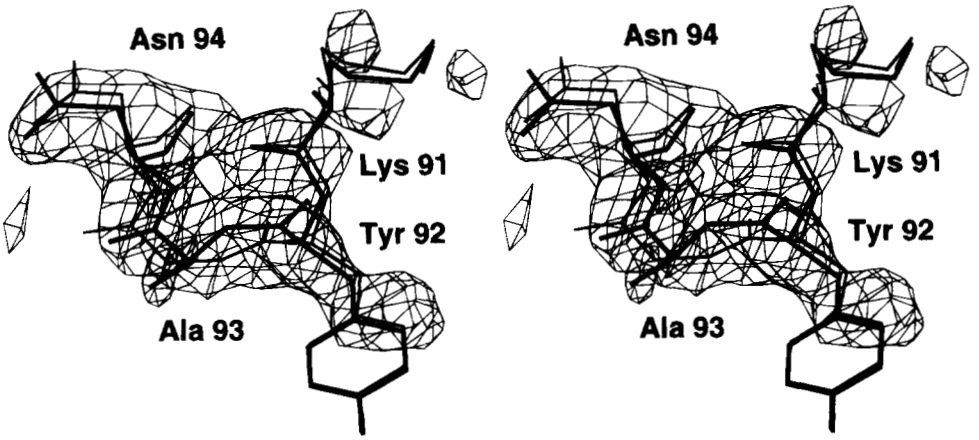

Fig. 2. Structure of residues 91–94 in the P93A ribonuclease. The refined structures containing a *cis* Tyr92–Ala93 peptide bond (thick line) and a *trans* Tyr92–Ala93 peptide bond (thin line) are shown with electron density ($2F_o - F_c$ with this region omitted from the model) contoured at 1.1 ρ_{rms}. The lack of clear electron density for the side chain of Tyr92 is due to the disorder in this region of the protein. Although this residue is not modeled well by any single conformation, the wild-type conformation shown here provides a reasonable fit.

residues 36–40 and 90–95, although the *cis* conformation of the peptide bond between residues 92 and 93 appears to be maintained in both average structures. Importantly, in the crystal structure of the C[40, 95]A variant, the position and mobility of the disulfide bond between Cys65 and Cys72 appears to be unaffected, which is consistent with the structural characterization of this variant by NMR (Laity et al., 1997). This observation supports the kinetic result that the structures of the intermediates in the two oxidative folding and reductive unfolding pathways of RNase A are conformationally distinct (Rothwarf et al., 1998; Li et al., 1995).

Methods: The preparations of the P93A and C[40, 95]A variants have been described elsewhere (Dodge & Scheraga, 1996; Laity et al., 1997). Both proteins were crystallized as described for a semisynthetic ribonuclease (Martin et al., 1987). Prior to data collection, CsCl was removed from the crystals as described for the semisynthetic enzyme. All crystallographic data sets were collected from single crystals at room temperature on an ADSC multiwire area detector system and processed using Scalepack (Otwinowski & Minor, 1997). Data collection for the P93A variant yielded 7,204 unique reflections between infinity and 2.3 Å (97% completeness, 5.0-fold redundancy) with an overall R_{meas} (Diederichs & Karplus, 1997) of 14.8% and an overall $R_{mrgd\text{-}F}$ (Diederichs & Karplus, 1997) of 12.3%. Data collection for the C[40, 95]A variant yielded 20,673 unique reflections between infinity and 1.6 Å (97% completeness, 3.2-fold redundancy) with an overall R_{meas} of 4.4% and an overall $R_{mrgd\text{-}F}$ of 7.9%. The starting model for both variants was that of the semisynthetic RNase A (Martin et al., 1987). After rigid-body refinement using the standard protocols in X-PLOR (Brünger, 1992b), difference electron density maps ($2F_o - F_c$ and $F_o - F_c$) were used to determine the position and extent of structural changes. In both variants, the region where the chain break was present in the semisynthetic enzyme had clear electron density indicating a return to the wild-type conformation. The models were adjusted manually using the program CHAIN (Sack, 1988), and the adjusted models were refined against all data between 10 Å and the nominal resolution limit using the conventional positional and restrained *B*-factor refinement protocols in X-PLOR. The P93A variant was refined to an *R*-factor of 21.0% and an R_{free} (Brünger, 1992a) of 27.2% (10–2.3 Å), while the C[40, 95]A variant was refined to an *R*-factor of 22.3% and an R_{free} of 27.2% (10–1.6 Å).

Acknowledgments: This work was supported in part by a research grant from the National Institute of General Medical Sciences of the National Institutes of Health (GM-24893). M.A.P. received financial support from a Hoechst Marion Roussel fellowship and an NIH Training Grant (GM08500). R.W.D. was an NIH Postdoctoral Fellow (1992–1995).

References

Brandts JF, Halvorson HR, Brennan M. 1975. Consideration of the possibility that the slow step in protein denaturation reactions is due to *cis-trans* isomerism of proline residues. *Biochemistry 14*:4953–4963.

Brünger AT. 1992a. Free R value: A novel statistical quantity for assessing the accuracy of crystal structures. *Nature 355*:472–475.

Brünger AT. 1992b. *X-PLOR*, Version 3.1. New Haven, Connecticut: Yale University Press.

Diederichs K, Karplus PA. 1997. Improved R-factors for diffraction data analysis in macromolecular crystallography. *Nat Struct Biol 4*:269–275.

Dodge RW, Scheraga HA. 1996. Folding and unfolding kinetics of the proline-to-alanine mutants of bovine pancreatic ribonuclease A. *Biochemistry 35*:1548–1559.

Garel JR, Baldwin RL. 1973. Both the fast and slow refolding reactions of ribonuclease A yield native enzyme. *Proc Natl Acad Sci USA 70*:3347–3351.

Houry WA, Rothwarf DM, Scheraga HA. 1994. A very fast phase in the refolding of disulfide-intact ribonuclease A: Implications for the refolding and unfolding pathways. *Biochemistry 33*:2516–2530.

Houry WA, Scheraga HA. 1996. The nature of the unfolded state of ribonuclease A: Effect of *cis-trans* X-Pro peptide bond isomerization. *Biochemistry 35*:11719–11733.

Juminaga D, Wedemeyer WJ, Garduño-Júarez R, McDonald MA, Scheraga HA. 1997. Tyrosyl interactions in the folding and unfolding of bovine pancreatic ribonuclease A: A study of tyrosine-to-phenylalanine mutants. *Biochemistry 36*:10131–10145.

Karplus PA. 1996. Experimentally observed conformation-dependent geometry and hidden strain in proteins. *Protein Sci 5*:1406–1420.

Laity JH, Lester CC, Shimotakahara S, Zimmerman DE, Montelione GT, Scheraga HA. 1997. Structural characterization of an analog of the major rate-determining disulfide folding intermediate of bovine pancreatic ribonuclease A. *Biochemistry 36*:12683–12699.

Li YJ, Rothwarf DM, Scheraga HA. 1995. Mechanism of reductive protein unfolding. *Nat Struct Biol* 2:489–494.

Martin PD, Doscher MS, Edwards BFP. 1987. The refined crystal structure of a fully active semisynthetic ribonuclease at 1.8-Å resolution. *J Biol Chem* 262:15930–15938.

Otwinowski Z, Minor W. 1997. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.

Rothwarf DM, Li YJ, Scheraga HA. 1998. Regeneration of bovine pancreatic ribonuclease A. Identification of two native-like three-disulfide intermediates involved in separate pathways. *Biochemistry* 37:3760–3766.

Sack JS. 1988. CHAIN—A crystallographic modeling program. *J Mol Graphics* 6:224–225.

Shimotakahara S, Rios CB, Laity JH, Zimmerman DE, Scheraga HA, Montelione GT. 1997. NMR structural analysis of an analog of an intermediate formed in the rate-determining step of one pathway in the oxidative folding of bovine pancreatic ribonuclease A: Automated analysis of ^{1}H, ^{13}C, and ^{15}N resonance assignments for wild-type and [C65S, C72S] mutant forms. *Biochemistry* 36:6915–6929.

Wlodawer A, Svensson LA, Sjölin L, Gilliland GL. 1988. Structure of phosphate-free RNase A refined at 1.26 Å. *Biochemistry* 27:2705–2717.

Zimmerman SS, Scheraga HA. 1976. Stability of *cis*, *trans*, and nonplanar peptide groups. *Macromolecules* 9:408–416.