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Contribution of a tyrosine side chain to ribonuclease A catalysis and stability

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Abstract

An intricate architecture of covalent bonds and noncovalent interactions appear to position the side chain of Lys 41 properly within the active site of bovine pancreatic ribonuclease A (RNase A). One of these interactions arises from Tyr 97, which is conserved in all 41 RNase A homologues of known sequence. Tyr 97 has a solvent-inaccessible side chain that donates a hydrogen bond to the main-chain oxygen of Lys 41. Here, the role of Tyr 97 was examined by replacing Tyr 97 with a phenylalanine, alanine, or glycine residue. All three mutant proteins have diminished catalytic activity, with the value of k_{cat} being perturbed more significantly than that of K_m. The free energies with which Y97F, Y97A, and Y97G RNase A bind to the rate-limiting transition state during the cleavage of poly(cytidylic acid) are diminished by 0.74, 3.3, and 3.8 kcal/mol, respectively. These results show that even though Tyr 97 is remote from the active site, its side chain contributes to catalysis. The role of Tyr 97 in the thermal stability of RNase A is large. The conformational free energies of native Y97F, Y97A, and Y97G RNase A are decreased by 3.54, 12.0, and 11.7 kcal/mol, respectively. The unusually large decrease in stability caused by the Tyr → Phe mutation could result from a decrease in the barrier to isomerization of the Lys 41–Pro 42 peptide bond.

Keywords: bovine pancreatic ribonuclease A; hydrogen bond; hydrophobic effect; prolyl peptide bond isomerization; protein stability; steady-state kinetics; thermal denaturation

Evolution has refined the structure of proteins so as to orient residues for function and stability. Still, the relative energetic contribution of hydrogen bonds and hydrophobic forces to protein structure remains an unresolved question in protein science. The ability to generate mutant proteins with single amino acid substitutions has greatly enhanced our understanding of the atomic basis for protein stability. Recent work on ribonuclease T1 (Pace et al., 1990; Shirley et al., 1992), T4 lysozyme (Matthews et al., 1987; Eriksson et al., 1992b; Matthews, 1993), λ repressor (Sauer et al., 1990; Lim et al., 1992), and other proteins has disclosed the contributions of particular noncovalent interactions to protein stability. Here, we explore the architecture of bovine pancreatic ribonuclease A (RNaseA; EC 3.1.27.5), which has already been the object of much seminal work in protein science.

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Abbreviations: MES, 4-morpholine-ethanesulphonic acid; poly(C), poly(cytidylic acid); RNase A, bovine pancreatic ribonuclease A; RNase T1, ribonuclease T1; T_m, midpoint of the thermal unfolding curve; U>v, uridine 2′,3′-cyclic vanadate.

RNase A catalyzes the cleavage of the P-$O_{5'}$ bond in RNA specifically after pyrimidine residues (Richards & Wyckoff, 1971). In the catalytic mechanism (Fig. 1A), His 12 acts as a general base that abstracts a proton from the 2′-hydroxyl group of a substrate molecule and thereby facilitates the attack of the 2′-oxygen on the phosphorus atom (Findlay et al., 1961; Roberts et al., 1969; Thompson & Raines, 1994). This attack proceeds in-line to displace the nucleoside (Usher et al., 1972). His 119 acts as a general acid that protonates the 5′-oxygen to facilitate its displacement with both products being released to solvent. Slow hydrolysis of the 2′,3′-cyclic phosphate occurs separately and resembles the reverse of transphosphorylation (Cuchillo et al., 1993; Thompson et al., 1994).

The structure of uridine 2′,3′-cyclic vanadate bound to RNase A has provided invaluable insight into the enzymatic reaction mechanism (Fig. 1B) (Wlodawer et al., 1983). U>v is a putative transition state analogue, as the transphosphorylation and hydrolysis reactions catalyzed by RNase A are likely to proceed through a transition state that contains significant pentavalent character. In the RNase A·U>v complex, the side chains of His 12 and His 119 are proximal to the vanadyl group, consistent with the mechanism in Figure 1A. In addition, two other side chains interact intimately with the vanadyl group. The side chain of Gln 11 donates a hydrogen bond to the O_{1V} nonbridg-

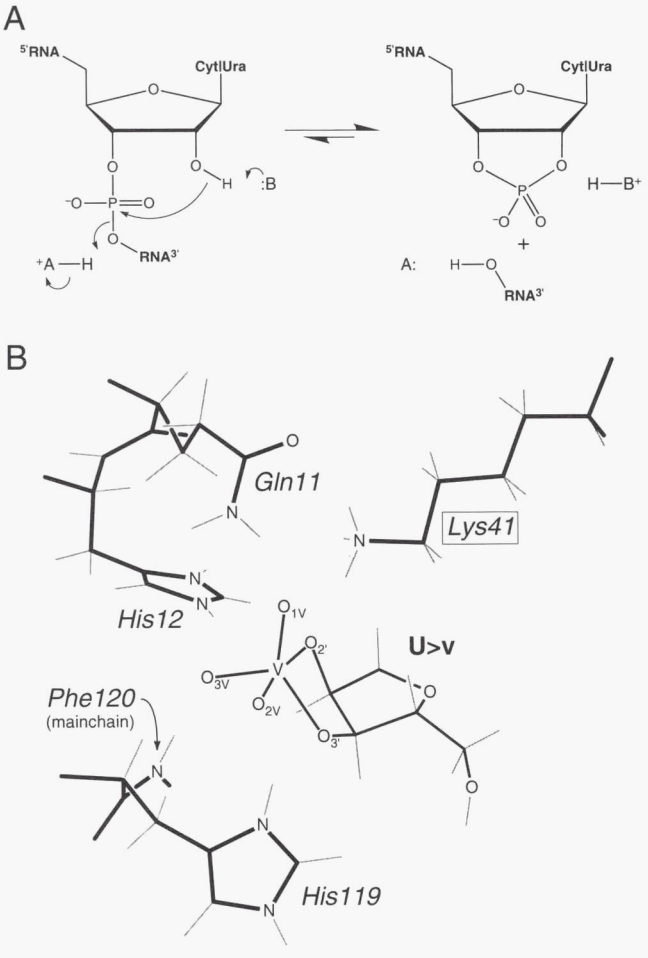

Fig. 1. A: Mechanism of the transphosphorylation reaction catalyzed by RNase A (Findlay et al., 1961). B is His 12; A is His 119. The side chain of Lys 41 (not shown) donates a hydrogen bond to the transition state during catalysis. **B:** Structure of the active site of RNase A bound to uridine 2′,3′-cyclic vanadate, a transition state analogue. The structure was refined at 2.0 Å from X-ray and neutron diffraction data collected from crystals grown at pH 5.3 (Wlodawer et al., 1983). The side chain of Phe 120 and the uracil base are not shown.

ing oxygen. The role of this residue is to prevent the nonproductive binding of substrate (delCardayré et al., 1995). The side chain of Lys 41 also donates a hydrogen bond to the vanadyl group.

A picture for the role of Lys 41 in catalysis has developed over the last 30 years. The importance of Lys 41 was discovered by chemical modification studies (Hirs et al., 1965; Murdock et al., 1966). Subsequent structural analyses led to the proposal that Lys 41 stabilizes selectively the chemical transition state for RNA cleavage (Alber et al., 1983). In the RNase A·U>v complex, the N_ζ of Lys 41 is 2.8 and 3.6 Å from the $O_{2'}$ bridging and O_{1V} nonbridging oxygens, respectively. Only recently, however, have heterologous gene expression systems been developed that allow for the revelation of structure-function relationships in RNase A by site-directed mutagenesis. Mutations of RNase A at position 41 compromise the ability of RNase A to catalyze the cleavage of RNA (Raines & Rutter, 1989; Raines, 1991; Trautwein et al., 1991; Laity et al., 1993; Tarragona-Fiol et al., 1993; Messmore et al., 1995). In addition, alkylation of K41C RNase A with various functional groups indicates that it is the hydrogen bond donated by the side chain of Lys 41 that stabilizes the transition state during catalysis (Messmore et al., 1995).

In folded proteins, a network of covalent bonds and noncovalent interactions lowers the conformational freedom of the main chain. In RNase A, this network appears to orient Lys 41 within the active site (Fig. 2). This orientation is critical because the side chain of Lys 41 in the RNase A·U>v complex is extended completely, a conformation necessary for N_ζ to reach a phosphoryl group bound in the active site. Adjacent to Lys 41, the side chain of Cys 40 participates in a disulfide bond with Cys 95 that links the first and third strands of the antiparallel β-sheet of RNase A. Replacing Cys 40 and Cys 95 with serine residues de-

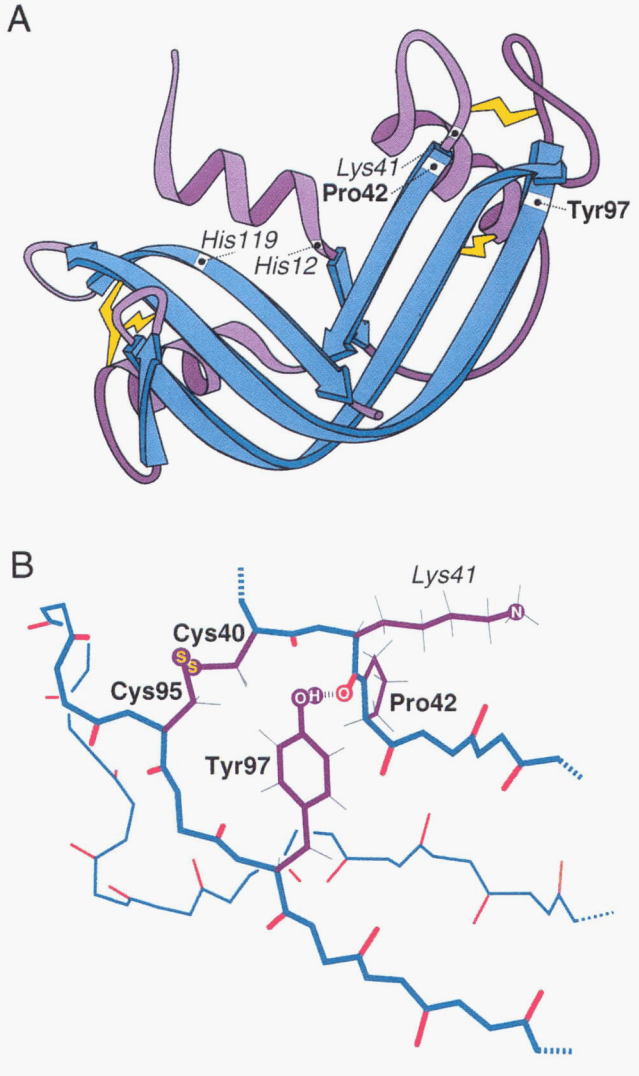

Fig. 2. A: Schematic three-dimensional structure of RNase A showing the location of Tyr 97, Pro 42, and active-site residues (italics) His 12, His 119, and Lys 41. **B:** Hydrogen bond formed between the side chain of Tyr 97 and oxygen of the Lys 41–Pro 42 peptide bond (Wlodawer et al., 1983). The only side chains shown are those of Cys 40, Lys 41, Pro 42, Cys 95, and Tyr 97. The main chain of the three-stranded β-sheet is shown from the face opposite to that in panel A.

creases the catalytic activity of RNase A by 20-fold and the T_m by 20 °C (Laity et al., 1993). The side chain of Tyr 97 donates a hydrogen bond to the main-chain oxygen of Lys 41, which participates in the prolyl peptide bond of Pro 42. This hydrogen bond (O-Oη distance = 2.4 Å, O-H-Oη angle = 162°), along with the rest of the side chain of Tyr 97, is buried completely within the interior of the folded protein. Spectroscopic studies of RNase A indicate that the hydroxyl group of Tyr 97 does not ionize readily (Eftink & Biltonen, 1987). Further, phylogenetic analyses shows that Tyr 97 is conserved in all 41 known homologues of RNase A, suggesting an important role for this residue (Beintema, 1987). We have changed Tyr 97 of RNase A to phenylalanine, alanine, and glycine, and used these mutant enzymes to dissect the contribution of the tyrosine side chain to catalysis and thermal stability.

Results

Steady-state kinetic parameters

The steady-state kinetic parameters for the cleavage of poly(C) by the wild-type and mutant RNase As are reported in Table 1. Replacing Tyr 97 with a phenylalanine, alanine, or glycine residue increases the value of K_m by ≤3.5-fold. This change is less than the 10-fold increase in K_m observed in K41A RNase A (J.M. Messmore & R.T. Raines, unpubl.). The mutations cause a more significant change in the value of k_{cat}. Although the value of k_{cat} for the Y97F enzyme is only twofold lower than that of the wild-type enzyme, the values of k_{cat} for the Y97A and Y97G enzymes are lower by 80-fold and 200-fold, respectively.

The decrease in k_{cat} and small increase in K_m are indicative of a significant reduction in ability to bind to the rate-limiting transition state. The values of k_{cat}/K_m for catalysis by Y97F, Y97A, and Y97G RNase A were observed to be 3.5-fold, 270-fold, and 580-fold lower than that of wild-type RNase A, respectively (Table 1). From these values, the free energy for binding to the rate-limiting transition state can be calculated with the equation:

$$\Delta\Delta G_{ts} = -RT \ln[(k_{cat}/K_m)_{\text{wild-type}}/(k_{cat}/K_m)_{\text{mutant}}]. \quad (1)$$

This analysis indicates that replacing Tyr 97 with phenylalanine, alanine, and glycine residues decreases binding to the rate-limiting transition state by 0.7, 3.3, and 3.8 kcal/mol, respectively (Table 1).

Table 1. *Steady-state kinetic parameters for the cleavage of poly(C) by wild-type, Y97F, Y97A, and Y97G RNase A*[a]

RNase A	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (10^6 $M^{-1}s^{-1}$)	$\Delta\Delta G_{ts}$ (kcal/mol)
Wild-type[b]	510 ± 10	0.034 ± 0.002	15 ± 1	–
Y97F	360 ± 10	0.080 ± 0.008	4.3 ± 0.3	−0.74
Y97A	6.4 ± 0.7	0.11 ± 0.03	0.056 ± 0.007	−3.3
Y97G	2.6 ± 0.7	0.10 ± 0.05	0.026 ± 0.005	−3.8

[a] Assays were performed in 0.10 M MES-HCl buffer, pH 6.0, containing NaCl (0.10 M). Values of k_{cat} and k_{cat}/K_m have been corrected for active enzyme concentrations at 25 °C using data in Figure 3. Values of K_m are based on nucleotide units in poly(C).

[b] From delCardayré & Raines (1994).

Thermal stability

The thermal stabilities of the wild-type and mutant RNase As are depicted in Figure 3. The inset in Figure 3 shows the reversible nature of the denaturation of Y97F RNase A. The denaturation of the other proteins is also reversible (data not shown). The thermal denaturation curves in Figure 3 highlight the difference in stability for all three mutants as compared to that of wild-type RNase A. All three mutants are significantly less stable than is wild-type RNase A. To determine the thermodynamic parameters for denaturation, the values of T and f_u in Figure 3 were fit to the equations (Becktel & Schellman, 1987):

$$\Delta G_m = \Delta H_m(1 - T/T_m) + \Delta C_p[T - T_m - T \ln(T/T_m)], \quad (2)$$

and

$$\Delta G_m = -RT \ln[f_u/(1 - f_u)], \quad (3)$$

where the subscript "m" refers to the midpoint of the thermal denaturation curve and f_u is the fraction of protein that is unfolded at a particular temperature. As reported in Table 2, removing the oxygen from the side chain of Tyr 97, as in Y97F RNase A, lowers the T_m by 10.1 °C. Replacing the phenol with a hydrogen, as in Y97A RNase A, lowers the T_m by an additional 24.2 °C. A subtle increase in stability of 1.0 °C is observed for the Y97G mutant over that of Y97A RNase A. The values of ΔH_m follow a similar trend.

The change in T_m caused by a small perturbation to a protein is related to the free energy of the perturbation ($\Delta\Delta G_m$) at the

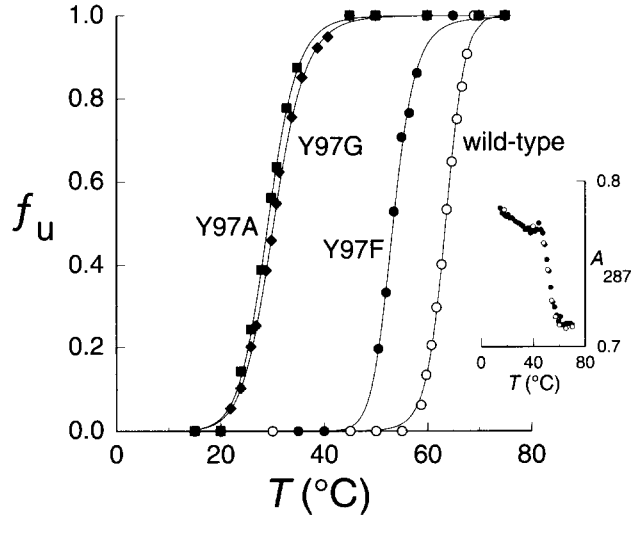

Fig. 3. Thermal denaturation of wild-type and mutant RNase A's. The fraction of unfolded protein (f_u) is shown at different temperatures for wild-type (○), Y97F (●), Y97A (■), and Y97G (◆) RNase A in 0.10 M sodium acetate buffer, pH 5.0, containing NaCl (0.10 M). **Inset:** Data for thermal denaturation (●) and renaturation (○) of Y97F RNase A in 0.10 M sodium acetate buffer, pH 5.0, containing NaCl (0.10 M).

Table 2. *Thermodynamic parameters for the denaturation of wild-type, Y97F, Y97A, and Y97G RNase A*[a]

RNase A	T_m (°C)	ΔT_m (°C)	ΔH_m (kcal/mol)	$\Delta\Delta G_m$ (kcal/mol)
Wild-type	63.3	–	118	–
Y97F	53.2	−10.1	97.8	−3.54
Y97A	29.0	−34.3	63.3	−12.0
Y97G	30.0	−33.3	58.8	−11.7

[a] Experiments were performed in 0.10 M sodium acetate buffer, pH 5.0, containing NaCl (0.10 M).

T_m of the unperturbed protein by the equation (Becktel & Schellman, 1987):

$$\Delta\Delta G_m = \Delta T_m \Delta S_m, \quad (4)$$

where

$$\Delta S_m = \Delta H_m / T_m, \quad (5)$$

and ΔS_m is the value for the wild-type protein. For RNase A, this value is calculated from Equation 5 and the values of T_m and ΔH_m in Table 2 to be $\Delta S_m = 2.85$ cal/(mol K). The values of $\Delta\Delta G_m$ calculated from Equation 4 are reported in Table 2. These values indicate that replacing Tyr 97 of RNase A with phenylalanine, alanine, and glycine residues decreases its conformational free energy by 3.54, 12.0, and 11.7 kcal/mol, respectively (Table 2).

Discussion

Role of Tyr 97 in catalysis

RNase A appears to have evolved to catalyze the cleavage of RNA rather than its hydrolysis (Cuchillo et al., 1993; Thompson et al., 1994). Moreover, its evolution is complete in that cleavage of its best substrates is limited by substrate desolvation rather than by changes in covalency (Thompson et al., 1995). Revealing the precise contribution of the active-site residues in Figure 1 to catalysis is an on-going effort of our research group (Thompson & Raines, 1994; delCardayré et al., 1995; Messmore et al., 1995).

Although Tyr 97 is far removed from the active site of RNase A (Fig. 2), truncating the side chain of Tyr 97 alters the steady-state kinetic parameters for catalysis (Table 1). This effect appears to arise in part from the role of Tyr 97 in orienting the side chain of Lys 41. Lys 41 participates in the cleavage of RNA by donating a hydrogen bond to the chemical transition state (Messmore et al., 1995). Thus, mutations that alter the position of Lys 41 within the active site of RNase A are expected to decrease the value of k_{cat}/K_m. We observed such an effect. The free energies lost for transition-state stabilization are $\Delta\Delta G_{ts} = -0.74$, -3.3, and -3.8 kcal/mol for the Y97F, Y97A, and Y97G RNase A, respectively. We therefore suggest that Tyr 97 aids catalysis by helping to orient the side chain of Lys 41.

Contribution of a hydrogen bond to protein stability

Removal of the hydrogen bond between Tyr 97 and the main-chain oxygen of Lys 41 is deleterious for the thermal stability of RNase A. As shown in Table 1, the change in thermal stability of Y97F RNase A is 3.54 kcal/mol less than that of the wild-type enzyme. This value is surprisingly large for a Tyr → Phe mutation. For example, Pace and coworkers found much smaller changes in thermal stability upon replacing any of the five tyrosine residues in RNase T1 with phenylalanine residues (Shirley et al., 1992). Their values for RNase T1 range from $\Delta\Delta G_m = 1.15$ to -2.03 kcal/mol. This difference between Tyr 97 of RNase A and the tyrosine residues in RNase T1 exists even though each side chain is at least 85% buried.

The contribution of a tyrosine hydroxyl group to protein stability could depend on the nature of the hydrogen bond acceptor. In RNase T1, each tyrosine residue donates a hydrogen bond to either another side chain or a typical (that is, non-prolyl) peptide bond. In RNase A, the hydroxyl group of Tyr 97 donates a hydrogen bond to the oxygen of the Lys 41–Pro 42 peptide bond. The *trans* isomer of a typical peptide bond is of much lower free energy than the *cis* isomer. In a prolyl peptide bond, however, the *trans* isomer has only slightly lower enthalpy ($\Delta H^\circ = -1.27$ kcal/mol) and equivalent entropy (Eberhardt et al., 1993). Further, isomerization of the Lys 41–Pro 42 peptide bond is of particular importance to RNase A. During protein folding, the isomerization of this bond is responsible for a slow kinetic phase (Kiefhaber et al., 1992; Kiefhaber & Schmid, 1992), which is absent in P42A RNase A (Dodge et al., 1994).

The rate of prolyl peptide bond isomerization is affected by environment. Previously, we had shown that the kinetic barrier to isomerization is increased by approximately 1.2 kcal/mol by the formation of a hydrogen bond to a main-chain oxygen (Eberhardt et al., 1992). This effect arises largely through ground state stabilization (Eberhardt, 1995). Removing such a hydrogen bond, as in Y97F RNase A, destabilizes the ground state of the *cis* and *trans* isomers and thereby allows a prolyl peptide bond to isomerize more rapidly. The loss of a hydrogen bond to the oxygen of a prolyl peptide bond could therefore result in the loss of conformational stability beyond that expected for the loss of a hydrogen bond to a typical peptide bond. This conclusion is consistent with conventional pictures of amide resonance (Pauling, 1948) and with the results of Matthews and coworkers, who found that a hydrogen bond to the *nitrogen* of a prolyl peptide bond *decreases* the barrier for prolyl peptide bond isomerization in wild-type dihydrofolate reductase (Texter et al., 1992).

Contribution of a benzyl group to protein stability

Removing the side chain of Phe 97 from Y97F lowers the melting temperature of RNase A dramatically. This decrease in stability can be attributed to the loss of hydrophobic surface area. Estimates from octanol phase transfer studies suggest that the contribution of the hydrophobic surface of a benzyl group to protein stability is $\Delta G_{tr} = 3$–5 kcal/mol (Fauchère & Pliska, 1983). In RNase A, the side chain of Phe 97 contributes significantly more. The data in Table 2 show that replacing this benzylic side chain with a methyl group (as in Y97A RNase A) decreases $\Delta\Delta G_m$ by 8.5 kcal/mol, and that replacing it with a hydrogen (as in Y97G RNase A) decreases $\Delta\Delta G_m$ by 8.2 kcal/mol.

The response of protein structures to cavity-creating mutations is not now predictable. Results from other systems indicate that the hydrophobic core is flexible and that side chains can reorganize in response to cavity-forming mutations (Eriksson et al., 1992a, 1992b, 1993; Lim et al., 1992; Buckle et al., 1993, 1996). Crystallographic studies are in progress to discern whether any reorganization occurs in the mutant RNase As (L.W. Schultz, E.S. Eberhardt, & R.T. Raines, unpubl.).

Manifestation of a protein-protein hydrogen bond in RNase A function

Folded proteins employ a network of covalent and noncovalent interactions that lower the conformational freedom of its amino acid residues. In RNase A, this network positions the side chain of Lys 41 properly within the active site. For example, the presence of an adjacent proline residue eliminates rotation about a dihedral angle (ϕ) in the main chain. The benefit of eliminating this rotational degree of freedom is partially offset, however, by the increased preference for the *cis* isomer about the prolyl peptide bond. The hydrogen bond formed by the main chain of Pro 42 and the side chain of Tyr 97 (which, like Pro 42, is a residue conserved by evolution) tethers the first and third β-strands (Fig. 2). Our results suggest that this hydrogen bond also lowers the conformational entropy of this region. Therefore, a significant role for the phenolic side chain of Tyr 97 could be to donate a hydrogen bond that stabilizes the *trans* isomer of the Lys 41–Pro 42 peptide bond and thereby locks the main chain of Lys 41 in a position that maximizes catalysis.

Materials and methods

Materials

Escherichia coli strain BL21(DE3) (F^- ompT r_B-m_B-) was from Novagen (Madison, Wisconsin). All enzymes for the manipulation of recombinant DNA were from Promega (Madison, Wisconsin). Reagents for DNA synthesis were from Applied Biosystems (Foster City, California), except for acetonitrile, which was from Baxter Healthcare (McGaw, Illinois). Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer, and purified using Oligo Purification Cartridges (Applied Biosystems). DNA was sequenced with a Sequenase 2.0 kit from United States Biochemicals (Cleveland, Ohio). DE52 anion exchange resin was from Whatman (Maidstone, England), and S-Sepharose cation exchange resin was from Pharmacia LKB (Piscataway, New Jersey). Poly(C) was from Sigma Chemical (St. Louis, Missouri) or from Midland Certified Reagent (Midland, Texas) and was precipitated from aqueous ethanol (70% v/v) before use. Wild-type RNase A was purchased from Sigma (St. Louis, Missouri). Bacto yeast extract, Bacto tryptone, Bacto peptone, and Bacto agar, were from Difco (Detroit, Michigan). Bacterial terrific broth (TB) (Tartof & Hobbs, 1987) contained (in 1 L) Bacto tryptone (12 g), Bacto yeast extract (24 g), glycerol (4 mL), KH_2PO_4 (2.31 g), and K_2HPO_4 (12.54 g). All media were prepared in distilled, deionized water and autoclaved. Ultraviolet and visible absorbance measurements were made with a Cary 3 spectrophotometer equipped with a Cary temperature controller. All other chemical reagents were of commercial reagent grade or better and were used without purification unless indicated otherwise.

Methods

Preparation of Tyr 97 mutants

Plasmid pBXR directs the expression of RNase A in *E. coli* (delCardayré et al., 1995). Oligonucleotide-mediated site-directed mutagenesis was performed on pBXR by the method of Kunkel et al. (1987) using *E. coli* strain CJ236. The TAC codon for Tyr 97 of RNase A was changed to TTT (phenylalanine), GCC (alanine), or GGC (glycine). Mutagenesis reaction mixtures were transformed into competent JM109 cells, and the DNA of the isolated transformants was analyzed by sequencing.

Production and purification of RNase A from E. coli

A glycerol freezer stock was prepared of *E. coli* strain BL21(DE3) harboring a mutated plasmid pBXR from a mid-log phase culture grown in LB medium containing ampicillin (400 μg/mL). This freezer stock was used to inoculate a starter culture (15 mL) of TB medium containing ampicillin (400 μg/mL). This culture was grown to mid-log phase (A = 0.5 at 600 nm) and used to inoculate a larger culture (1 L) of the same medium containing ampicillin (400 μg/mL). The inoculated culture was shaken (250 rpm) at 37 °C until it reached late log phase (A = 1.9 at 600 nm), and was then induced to express the cDNA that codes for RNase A by the addition of IPTG (2 mM). Shaking at 37 °C was continued for 4 h, before the cells were harvested by centrifugation for 10 min at 5,000 × g.

The cell pellet was resuspended in cell lysis buffer (250 mL), which was 20 mM Tris-HCl buffer, pH 7.8, containing urea (6 M) and EDTA (1 mM), and the suspension was shaken for 20 min at 37 °C. The suspension was then centrifuged for 15 min at 30,000 × g, and the resulting pellet was resuspended in solubilization buffer (250 mL), which was 20 mM Tris-HCl buffer, pH 7.8, containing 6 M urea, 0.4 M NaCl, 20 mM DTT, and 1 mM EDTA, and the suspension was shaken for 20 min at 37 °C. This suspension was then centrifuged for 15 min at 30,000 × g. Reduced DTT (0.22 g) was added to the supernatant and the resulting solution was stirred at room temperature for 10 min. Glacial acetic acid was added to lower the pH to 5.0, and the resulting solution was dialyzed exhaustively against 20 mM Tris-AcOH buffer, pH 5.0, containing 0.1 M NaCl. The insoluble material that accumulated during dialysis was removed by centrifugation. The soluble fraction was then reoxidized by exhaustive dialysis (>24 h) against refolding buffer, which was 50 mM Tris-AcOH buffer, pH 7.8, containing 0.1 M NaCl, 1 mM reduced glutathione, and 0.2 mM oxidized glutathione. The refolded sample was then dialyzed exhaustively against 20 mM Tris-AcOH buffer, pH 8.0. The dialyzed sample was passed through a column (15 cm × 4.9 cm^2) of DE-52 anion exchange resin equilibrated with the same buffer. The flow-through was loaded onto an FPLC mono-S cation exchange column (15 cm × 1.8 cm^2) that had been equilibrated with 20 mM Tris-AcOH buffer, pH 8.0, and the loaded column was washed with the same buffer (100 mL). RNase A was eluted with a linear gradient of NaCl (0.0–0.35 M) in Tris-AcOH buffer, pH 8.0. Fractions were collected and assayed for ribonuclease activity. The purity of active fractions was assessed by SDS-PAGE. Fractions containing RNase A of >95% purity were pooled and concentrated by ultrafiltration on an Amicon YM10 membrane. The concentrate was loaded onto an FPLC HiLoad™ 26/60 Superdex™ 75 gel filtration column that had been equilibrated with

50 mM sodium acetate buffer, pH 5.0, containing 0.10 M NaCl and 0.02% (w/v) NaN_3. The column was eluted with the same buffer, and the fractions corresponding to monomeric RNase A were pooled and characterized. Concentrations of mutant RNase As were determined by a dye-binding assay (Bradford, 1976) using a kit from Bio-Rad (Hercules, California). A standard curve was generated from solutions of wild-type RNase A by assuming that $A = 0.72$ at 277.5 nm for a 1.0 mg/mL solution (Sela et al., 1957).

Steady-state kinetic analyses

The cleavage of poly(C) was monitored by the change in its ultraviolet absorbance. Concentrations of mononucleotide units in poly(C) were determined by ultraviolet absorption at pH 7.8 using $\epsilon = 6{,}200\ M^{-1}cm^{-1}$ at 268 nm. The difference molar absorptivity ($\Delta\epsilon$) between a mononucleotide unit in the polynucleotide substrate and the mononucleotide 2′,3′-cyclic phosphate product was 2,380 $M^{-1}cm^{-1}$ at 250 nm (delCardayré et al., 1995). All assays were performed at 25 °C in 0.10 M MES-HCl buffer, pH 6.0, containing 0.10 M NaCl, 10 μM–0.4 mM poly(C), and an appropriate amount of enzyme. Values of k_{cat}, K_m, and k_{cat}/K_m were determined from the initial velocity data with the program HYPERO (Cleland, 1979).

Thermal denaturation

As RNase A is denatured, its six tyrosine residues become exposed to solvent and its molar absorptivity at 287 nm decreases significantly (Hermans & Scheraga, 1961). The thermal stability of RNase A proteins was assessed by monitoring by the change of absorbance at 287 nm with temperature (Pace et al., 1989). Solutions (0.8 mL) of protein (1.0–3.0 mg/mL) were prepared in 0.10 M sodium acetate buffer, pH 5.0, containing 0.10 M NaCl. The absorbance at 287 nm was recorded as the temperature was increased from 10 to 95 °C in 1 °C increments with a 5-min equilibration at each temperature. Similarly, the absorbance at 287 nm was also recorded as the temperature was decreased from 95 to 10 °C. Values of T_m and ΔH_m were determined from Equations 2 and 3 by nonlinear regression analyses of the denaturation curves using the program SIGMA PLOT 4.16.

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