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Ribonuclease A: Revealing Structure−Function Relationships with Semisynthesis

June M. Messmore, Dana N. Fuchs, and Ronald T. Raines*

Contribution from the Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706-1569

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Abstract: Bovine pancreatic ribonuclease A (RNase A) catalyzes the cleavage of P−O$^{5'}$ bonds in RNA. Structural analyses had suggested that the active-site lysine residue (Lys41) may interact preferentially with the transition state for covalent bond cleavage, thus facilitating catalysis. Here, site-directed mutagenesis and semisynthesis were combined to probe the role of Lys41 in the catalysis of RNA cleavage. Recombinant DNA techniques were used to replace Lys41 with an arginine residue (K41R) and with a cysteine residue (K41C), which had the only sulfhydryl group in the native protein. The value of k_{cat}/K_m for cleavage of poly(C) by K41C RNase was 10^5-fold lower than that by the wild-type enzyme. The sulfhydryl group of K41C RNase A was alkylated with five different haloalkylamines. The values of k_{cat}/K_m for the resulting semisynthetic enzymes and K41R RNase A were correlated inversely with the values of pK_a for the side chain of residue 41. Further, no significant catalytic advantage was gained by side chains that could donate a second hydrogen bond. These results indicate that residue 41 donates a single hydrogen bond to the rate-limiting transition state during catalysis.

Introduction

Illuminating the role of individual amino acid residues in enzymatic catalysis was made less problematic by the introduction of oligonucleotide-mediated site-directed mutagenesis.[1] Since then, biological chemists have been able to exchange any one of the 20 naturally-incorporated amino acid residues for any other. Still, the common, natural amino acids display limited functionality. We report the use of *cysteine elaboration* to introduce nonnatural amino acid residues at a specific position in the active site of bovine pancreatic ribonuclease A (RNase A; E. C. 3.1.27.5).[2−5] The advantage of such a strategy here is that the incorporation of nonnatural functional groups allows

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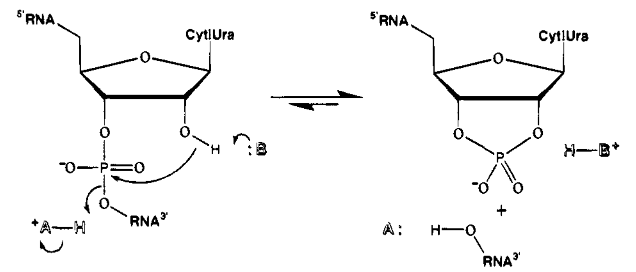

Figure 1. Mechanism for the cleavage of RNA as catalyzed by RNase A.[6] B is histidine 12; A is histidine 119.[7]

for greater variation in side-chain length and pK_a than does the use of common amino acid residues.

RNase A efficiently catalyzes the cleavage of P–O$^{5'}$ bonds in RNA, specifically after pyrimidine residues (Figure 1).[8] This enzyme has been the object of landmark work on enzymology; on the folding, structure, and chemistry of proteins; and on molecular evolution.[9] We have combined site-directed mutagenesis with semisynthesis to probe the role of the lysine residue at position 41 (Lys41). This residue was known from structural,[10] chemical modification,[11] and site-directed mutagenesis[12] studies to be important for catalysis, but its precise role in catalysis had been unclear. Our results indicate that residue 41 donates a single hydrogen bond to the chemical transition state during RNA cleavage.

Results and Discussion

We used recombinant DNA techniques to produce an RNase A mutant in which Lys41 was changed to either a cysteine or an arginine residue. The change to cysteine introduces a solvent accessible sulfhydryl group to the native protein. (The other eight cysteine residues form four disulfide bonds in native RNase A.) This sulfhydryl group was then alkylated with five haloalkylamines. In each of the resulting semisynthetic enzymes, residue 41 contains a nitrogen separated from the main chain by either 4 atoms (as in lysine) or 5 atoms (as in arginine). We then determined the ability of the wild-type, mutant, and semisynthetic ribonucleases to catalyze the cleavage of polycytidylic acid [poly(C)].

The values of the steady-state kinetic parameters for cleavage of poly(C) by our ribonucleases are given in Table 1. The second-order rate constant, k_{cat}/K_m, is proportional to the association constant of an enzyme and the rate-limiting transition state during catalysis.[13] We define the related free energy difference, $\Delta\Delta G^{\ddagger}$, as the loss in ability of each mutant and semisynthetic ribonuclease to bind to the rate-limiting transition state during catalysis. As shown in Table 1, dramatic differences are observed in the values of k_{cat}/K_m and $\Delta\Delta G^{\ddagger}$, indicating that the mere presence of an alkylamine is not enough to effect efficient catalysis.

Structural Implications. The result of modifying the cysteine-containing mutant protein (K41C RNase A) with bromoethylamine is an enzyme quite closely related to wild-type RNase A. Yet, the value of k_{cat}/K_m for catalysis by K41 *S*-(aminoethyl)cysteine RNase A is only 8% that of the wild-type enzyme. The difference in the two proteins must lie in the differences between a thioether group and a methylene group. Although the angles of C–S–C bonds tend to be more acute than those of C–CH$_2$–C bonds, this difference is offset by the greater length of C–S bonds.[3g,14] Molecular modeling indicates that the primary amine groups in *S*-(aminoethyl)cysteine and lysine can be superimposed to within 0.1 Å. A more significant difference between *S*-(aminoethyl)cysteine and lysine is their relative preference for gauche rather than anti torsion angles.[15] The anti conformation of CC–CC bonds is favored by approximately 0.8 kcal/mol in model compounds.[16] Indeed, the average torsion angle in the side chain of Lys41 is (175 ± 3)° in the complex of RNase A with uridine 2',3'-cyclic vanadate (U>v), a putative transition state analog (Figure 2). In contrast to CC–CC bonds, the gauche conformation of CS–CC bonds is favored by 0.05–0.20 kcal/mol.[17] Molecular modeling indicates that the CS–CC bond of an *S*-(aminoethyl)cysteine residue at position 41 can be in the gauche conformation without disturbing the structure of the native protein. Thus, the thioether side chains at position 41 are likely to be less rigid and extended than are the alkyl side chains. We therefore surmise that catalysis by the *S*-(aminoethyl)cysteine enzyme is not as efficient as that by wild-type RNase A because of the energetic cost of fixing a thioether in the all-anti conformation.

Catalytic efficiency depends on the length of the side chain of residue 41. RNase A variants that present an amino group at the end of a side chain longer than that of lysine are more active catalysts than is unmodified K41C RNase A. Thus, additional length is tolerated in the active site. Still, enzymes in which an amino group at position 41 is separated from the main chain by 4 atoms are more active than are those in which

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Table 1. Steady-State Kinetic Parameters for the Cleavage of Poly(C) by Wild-Type, Mutant, and Semisynthetic Ribonucleases

residue 41	side chain	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G^{\ddagger}$ [a] (kcal/mol)	pK_a of side chain *NH* [b]
cysteine	$\sim CH_2-SH$	0.026 ± 0.004	0.36 ± 0.12	73 ± 15	6.8	
lysine (wild-type)	$\sim CH_2CH_2CH_2CH_2NH_3^+$	604 ± 47	0.091 ± 0.022	$(6.5 \pm 1.2) \times 10^6$	0.0	10.6
S-(aminoethyl)cysteine	$\sim CH_2-S-CH_2CH_2NH_3^+$	43 ± 3	0.075 ± 0.016	$(5.2 \pm 1.0) \times 10^5$	1.5	10.6
S-acetamidinocysteine	$\sim CH_2-S-CH_2C(NH_2)NH_2^+$	11.0 ± 0.4	0.041 ± 0.006	$(2.6 \pm 0.3) \times 10^5$	1.9	12.5
S-(carbamoylmethyl)cysteine	$\sim CH_2-S-CH_2C(O)NH_2$	0.074 ± 0.007	0.25 ± 0.05	301 ± 28	5.9	15.2
S-((trimethylamino)ethyl)cysteine	$\sim CH_2-S-CH_2CH_2N(CH_3)_3^+$	nd	nd	<230[c]	>6.1[c]	
S-(aminopropyl)cysteine	$\sim CH_2-S-CH_2CH_2CH_2NH_3^+$	12.9 ± 0.4	0.12 ± 0.01	$(1.1 \pm 0.1) \times 10^5$	2.4	10.6
arginine	$\sim CH_2CH_2CH_2NHC(NH_2)NH_2^+$	4.4 ± 0.3	0.091 ± 0.016	$(4.8 \pm 0.6) \times 10^4$	2.9	13.7

[a] $\Delta\Delta G^{\ddagger} = RT\ln[(k_{cat}/K_m)^{\text{wild-type}}/(k_{cat}/K_m)]$. [b] Based on aqueous solutions of the model compounds: lysine, *S*-(aminoethyl)cysteine, *S*-(aminopropyl)cysteine, $CH_3CH_2CH_2CH_2NH_3^+$ (Hall, H. K., Jr. *J. Am. Chem. Soc.* **1957**, *79*, 5441–5444); *S*-acetamidinocysteine, $CH_3C(NH_2)NH_2^+$ and arginine, $H_2NC(NH_2)NH_2^+$ (Albert, A. R.; Goldacre, R.; Phillips, J. *J. Chem. Soc.* **1948**, *3*, 2240–2249); *S*-(carbamoylmethyl)cysteine, $CH_3C(O)NH_2$ (Bordwell, F. G. *Acc. Chem. Res.* **1988**, *21*, 456–463). [c] Assuming $K_m \geq 0.1$ mM.

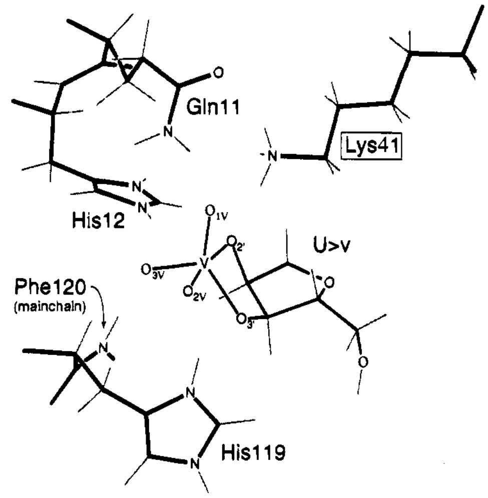

Figure 2. Structure of the active site of RNase A bound to uridine 2′,3′-cyclic vanadate. The structure was refined at 2.0 Å from X-ray and neutron diffraction data collected from crystals grown at pH 5.3. The side chain of phenylalanine 120 and the uracil base are not shown.

the separation is 5 atoms. This result could arise from the additional conformational entropy or unfavorable torsion angles of longer side chains.

No significant advantage is gained if residue 41 can donate a second hydrogen bond. Guanidino and acetamidino groups have the potential to interact simultaneously with more than one oxygen of a phosphoryl group. For example, a guanidino group is used to bind phosphoryl groups by the HIV-1 Tat protein[18] and by artificial receptors.[19] Further, in Staphylococcal nuclease and ribonucleases of the T1 family, an arginine appears to play the role of Lys41 in RNase A.[20] We have replaced Lys41 with an arginine residue and an *S*-acetamidino residue, which is a short analog of arginine. The values of $\Delta\Delta G^{\ddagger}$ for these enzymes are greater than those for the analogous enzymes with only a primary amino group in the side chain of position 41. Thus, one hydrogen bond appears to be enough to effect efficient catalysis. This result is consistent with the crystalline complex of RNase A with U>v (Figure 2). The vanadyl group in U>v is an approximate trigonal bipyramid with two nonbridging equatorial oxygens, $O_{1v.c.}$ and $O_{3v.c.}$. Oxygen $O_{1v.c.}$ accepts a hydrogen bond from the side chain of glutamine 11 while $O_{3v.c.}$ accepts a hydrogen bond from the main chain of phenylalanine 120. Only $O_{1v.c.}$ is in position to accept a hydrogen bond from Lys41.

Mechanistic Implications. The catalytic role most commonly attributed to Lys41 is to stabilize the excess negative charge built up on the nonbridging phosphoryl oxygens during RNA cleavage. Charge buildup could occur in a pentacoordinate transition state (or phosphorane intermediate) when the 2′ hydroxyl group attacks the phosphorous, on the way to displacing the 5′ nucleoside. It has been assumed that this stabilization occurs by Coulombic interactions.[12c,21] But, it has also been proposed recently that the stabilization occurs by way of a short, strong hydrogen bond involving the partial transfer of a proton from Lys41.[22]

The salient features of a lysine residue are its positive charge and its capacity to donate hydrogen bonds. In general, it is not a simple matter to distinguish the contribution of Coulombic forces from that of hydrogen bonds. What follows is the simplest explanation that is consistent with the data in Table 1.

The distinction between hydrogen bonds and Coulombic forces is evident nowhere more than in a comparison of the *S*-((trimethylamino)ethyl)cysteine enzyme, which possesses a terminal positive charge but no ability to donate a hydrogen bond, and the *S*-(aminoethyl)cysteine enzyme, which has both an N–H for potential hydrogen bond donation and a positive charge. The low catalytic activity of the *S*-((trimethylamino)ethyl)cysteine enzyme argues strongly against the efficacy of Coulombic forces in transition state stabilization. All else being equal, the energy of a charge–charge interaction diminishes only as the inverse of distance. The presence of the three methyl groups is likely to increase the distance between the positive charge on the side chain and the phosphoryl oxygens, relative to that in the *S*-(aminoethyl)cysteine enzyme. Still, the methyl groups can be accommodated in the vicinity of the phosphoryl oxygens without imposing any structural perturbations on the protein (Figure 2), and the increased distance is unlikely to be large enough to cause the observed > 10^4-fold reduction in k_{cat}/K_m.

The strength of a hydrogen bond is expected to correlate inversely with the pK_a of the proton being donated, inasmuch as hydrogen bonding involves some extent of proton transfer.[23] As shown in Table 1, increases in pK_a do indeed correspond to increases in $\Delta\Delta G^{\ddagger}$ for semisynthetic enzymes having side chains of comparable length. For those semisynthetic enzymes in

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which side chain lengths are comparable to lysine, the correlation is, however, nonlinear. This lack of linearity could arise because the pK_a of each side chain depends on its particular environment in the native protein. For example, the pK_a of Lys41 has been determined to be near 9.0,[24] rather than 10.6 as is listed for the butylammonium ion in Table 1. Different side chains may be affected to different extents. Another, perhaps more significant, source of nonlinearity is that charged species tend to participate in stronger hydrogen bonds than do uncharged species. This phenomenon has been observed for proteins[25] as well as small molecules, including amines.[26] Comparing semisynthetic enzymes with side chains that are isosteric but differ in formal charge should reveal any such tendency. For example, in the *S*-acetamidinocysteine and *S*-(carbamoylmethyl)-cysteine enzymes, the side chains at position 41 are identical except for one of the two heteroatoms attached to the terminal carbon. Yet, the difference in the ability of these two isologous side chains to bind to the transition state is greater than that expected from their acidities alone. Here, the hydrogen bond donated from a charged amidine is 4 kcal/mol stronger than is that from an uncharged amide. This value is consistant with other data on the relative strengths of charged and uncharged hydrogen bonds in protein–ligand interactions.[25] Finally, it is noteworthy that although the *S*-(carbamoylmethyl) side chain lacks a formal charge and has a relatively high pK_a, it still contributes (albeit modestly) to catalysis. This result provides further evidence of the importance to catalysis of a hydrogen bond donated by residue 41.

Conclusion

Site-directed mutagenesis followed by chemical modification has enabled us to study related enzymes that have more subtle changes in their active sites than would have been possible with site-directed mutagenesis alone. The correlation of high values of k_{cat}/K_m with low values of side-chain pK_a and the low activity of the *S*-((trimethylamino)ethyl)cysteine enzyme support a model in which the role of lysine 41 in catalysis by RNase A is to donate a hydrogen bond. Further, catalysis is not enhanced by the presence of a side chain at position 41 that can donate a second hydrogen bond. Finally, kinetic data are consistent with thioether side chains being less extended than alkyl side chains—lysine is *not* equivalent to *S*-(aminoethyl)cysteine.

Experimental Section

Materials. Bromoethylamine·HBr, bromopropylamine·HBr, iodoacetamide, and bromoethyltrimethylamine·HBr were from Sigma Chemical (St. Louis, MO), and were used without further purification. Chloroacetonitrile and other chemicals used in the synthesis of chloroacetamidine·HCl were from Aldrich Chemical (Milwaukee, WI) and were used without further purification.

Chloroacetamidine·HCl was synthesized from chloroacetonitrile and ammonium chloride as described by Schaefer and Peters.[27] Briefly, $NaOCH_3$ was added to a solution of chloroacetonitrile in MeOH. A stoichiometric amount of ammonium chloride was then added to the solution of the imidate. Solvent was removed under vacuum, and the product was washed several times with diethyl ether and dried under vacuum. The integrity and purity of the chloroacetamidine·HCl was confirmed by ^1H NMR (CD_3OD; 4.85 ppm relative to tetramethylsilane, s, CH_2), mass spectroscopy (EI, *m/e* 92; calcd for $C_2H_5ClN_2$ 92.0141), and melting point determination (92–94 °C; uncorrected). Chloroacetamidine·HCl was found to be stable under vacuum for at least 1 week.

Enzyme Preparation. Mutations in the cDNA that codes for RNase A were made by the method of Kunkel[28] using oligonucleotides AAGGTGTTAACTGGCCTGCATCGATC (for K41R) and AAAGGTGTTAACTGGACAGCATCGATC (for K41C). Mutant cDNA's were expressed in *Escherichia coli* under the control of the T7 RNA polymerase promoter, and the resulting proteins were refolded and purified as described.[29] The behavior of the K41C enzyme during FPLC suggests that the four native disulfide bonds form in high yield in this mutant enzyme. After purification, the new sulfhydryl group in the K41C enzyme was protected from inadvertant air oxidation by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).

Prior to alkylation of K41C RNase A, the sulfhydryl group of Cys41 was deprotected by treating the enzyme with dithiothreitol (0.1 mM) for 25 min at 25 °C, or until 1 equiv of 5-thio-2-nitrobenzoic acid had been released. Deprotected K41C RNase A was added to a freshly prepared solution of haloalkylamine (0.1 M) in 0.2 M Tris·HCl buffer, pH 8.3, and the resulting solution was incubated for 3 h at 30 °C. Semisynthetic enzymes were separated from any unreacted or undeprotected enzyme by cation exchange FPLC (Mono S column; Pharmacia, Piscataway, NJ) using a linear gradient of NaCl (0–200 mM) in 50 mM HEPES buffer, pH 7.7. The isolated yield of each semisynthetic enzyme was at least 50%.

The following observations indicate that alkylation was specific for Cys41. First, exposure of wild-type RNase A to the reaction conditions did not change its catalytic activity, indicating that fortuitous alkylation had not occurred near the enzymic active site. Second, no semisynthetic enzyme had a retention time during cation exchange FPLC that was greater than that of wild-type RNase A, indicating that no more than one additional positive charge had been introduced into K41C RNase A.

An acetamidino group can suffer hydrolysis to form an amide. To ascertain the stability of *S*-acetamidinocysteine RNase A, this enzyme was analyzed by cation exchange FPLC 10 days after it had been isolated and 8 days after its kinetic parameters had been determined. Then, the enzyme still appeared to be >95% pure.

Enzymatic Assays. Poly(C) was from Sigma Chemical or Midland Reagent (Midland, TX) and was purified by precipitation from aqueous ethanol (70% v/v). Assays of poly(C) cleavage were performed at 25 °C in 0.1 M Mes-HCl buffer, pH 6.0, containing NaCl (0.1 M). Cleavage of poly(C) was monitored by UV absorption using $\Delta\epsilon = 2380\ M^{-1}\ cm^{-1}$ at 250 nm.[8c] Steady-state kinetic parameters were determined by fitting initial velocity data to a hyperbolic curve using the program HYPERO.[30]

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