Ribonuclease A: Revealing Structure–Function Relationships with Semisynthesis

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Received October 24, 1994

Abstract: Bovine pancreatic ribonuclease A (RNase A) catalyzes the cleavage of P–O5 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. 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). The advantage of such a strategy here is that the incorporation of nonnatural functional groups allows


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Assemblies, Vol. 41 donates a single hydrogen bond to the chemical transition charged in vitro. For leading references, see: Comish, A mutant in which Lys41 was changed to either a cysteine or residues by using stop-codon suppressor tRNAs that have been tagged with semisynthesis to probe the role of the lysine residue at position 41 (Lys41). This residue was known from structural, chemical modification, and site-directed mutagenesis with semisynthesis to probe the role of the lysine residue at position 41. This residue was known from structural, chemical modification, and site-directed mutagenesis 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 poly-cytidylic 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. We define the related free energy difference, $\Delta \Delta G^*$, 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 $\Delta \Delta G^*$, 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 bromoethylyamine is an enzyme quite closely related to wild-type RNase A. Yet, the value of $k_{cat}/K_m$ for catalysis by K41S-(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-CH2-C bonds, this difference is offset by the greater length of C-S bonds. 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. The anti conformation of CC-C bonds is favored by approximately 0.8 kcal/mol in model compounds. 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>ν), a putative transition state analog (Figure 2). In contrast to CC-C bonds, the gauche conformation of CS-CC bonds is favored by 0.05-0.20 kcal/mol. Molecular modeling indicates that the CS-C 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 those in which

(5) Several groups have introduced nonnatural amino acids into specific sites of proteins by using stop-codon suppressor tRNAs that have been charged in vitro. For leading references, see: Cornish, V. W.; Schultz, P. G. Curr. Opin. Struct. Biol. 1994, 4, 601-607. We chose a semisynthetic route so as to generate large amounts of protein at little cost, and thereby allow for future structural analyses.


(8) For recent work on the energetics and specificity of the reaction in Figure 1, see: (a) Thompson, J. E.; Venegas, F. D.; Raines, R. T. Biochemistry 1994, 33, 7408-7414. (b) Thompson, J. E.; Kutaletadze, T. G.; Schugar, M. C.; Venegas, F. D.; Messmore, J. M.; Raines, R. T. Bioorg. Chem. Submitted for publication. (c) delcardayrk, S. B.; Raines, R. T. Biochemistry 1994, 33, 6031-6037. (d) delcardayrk, S. B.; Raines, R. T. J. Mol. Biol. In press.


Structural Function Relationships in Ribonuclease A

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

<table>
<thead>
<tr>
<th>residue 41</th>
<th>side chain</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>$\Delta\Delta G^\circ$ (kcal/mol)</th>
<th>$pK_a$ of side chain NH$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>cysteine</td>
<td>$\mathrm{CH}_3\mathrm{S}$H</td>
<td>0.026 ± 0.004</td>
<td>0.36 ± 0.12</td>
<td>73 ± 15</td>
<td>6.8</td>
<td>5.0</td>
</tr>
<tr>
<td>lysine (wild-type)</td>
<td>$\mathrm{CH}_3\mathrm{CH}_2\mathrm{CH}_2\mathrm{NH}_2^+$</td>
<td>604 ± 47</td>
<td>0.091 ± 0.022</td>
<td>$(6.5 \pm 1.2) \times 10^3$</td>
<td>0.0</td>
<td>10.6</td>
</tr>
<tr>
<td>S-(aminooethyl)cysteine</td>
<td>$\mathrm{CH}_2=\mathrm{SH}$</td>
<td>43 ± 3</td>
<td>0.075 ± 0.016</td>
<td>$(5.2 \pm 1.0) \times 10^3$</td>
<td>1.5</td>
<td>10.6</td>
</tr>
<tr>
<td>S-acetamidocysteine</td>
<td>$\mathrm{CH}_2=\mathrm{S}-\mathrm{CH}_2\mathrm{NH}_2$</td>
<td>11.0 ± 0.4</td>
<td>0.041 ± 0.006</td>
<td>$(2.6 \pm 0.3) \times 10^3$</td>
<td>1.9</td>
<td>12.5</td>
</tr>
<tr>
<td>S-(carbamoylmethyl)cysteine</td>
<td>$\mathrm{CH}_2=\mathrm{S}-\mathrm{CH}_2\mathrm{O}\mathrm{NHO}_2$</td>
<td>0.074 ± 0.007</td>
<td>0.25 ± 0.05</td>
<td>301 ± 0.28</td>
<td>5.9</td>
<td>15.2</td>
</tr>
<tr>
<td>S-(trimethylamino)ethyl)cysteine</td>
<td>$\mathrm{CH}_2=\mathrm{S}-\mathrm{CH}_2\mathrm{N(CH}_2\mathrm{)}_3\mathrm{H}_2$</td>
<td>nd</td>
<td>nd</td>
<td>&lt;230</td>
<td>&gt;6.1</td>
<td></td>
</tr>
<tr>
<td>S-(aminopropyl)cysteine</td>
<td>$\mathrm{CH}_2=\mathrm{S}-\mathrm{CH}_2\mathrm{CH}_2\mathrm{NH}_2$</td>
<td>12.9 ± 0.4</td>
<td>0.12 ± 0.01</td>
<td>$(1.1 \pm 0.1) \times 10^4$</td>
<td>2.4</td>
<td>10.6</td>
</tr>
<tr>
<td>arginine</td>
<td>$\mathrm{CH}_2=\mathrm{S}-\mathrm{CH}_2\mathrm{NH}_2\mathrm{C}(\mathrm{NH}_2\mathrm{)}_2\mathrm{H}_2$</td>
<td>4.4 ± 0.3</td>
<td>0.091 ± 0.016</td>
<td>$(4.8 \pm 0.6) \times 10^4$</td>
<td>2.9</td>
<td>13.7</td>
</tr>
</tbody>
</table>

\[ \Delta\Delta G^\circ = R\ln\left(k_{cat}/K_m\right) \](a)


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 phosphorus, on the way to displacing the 5' nucleoside. It has been assumed that this stabilization occurs by Coulombic interactions. (b) 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. (c)

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 bond 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-(aminooethyl)cysteine enzyme, which has both a terminal positive charge and the ability to donate a hydrogen bond. The low catalytic activity of the S-(trimethylamino)ethyl)cysteine enzyme argues strongly against the efficacy of Coulombic forces in establishing transition state stabilization. All else being equal, the energy of a charge–charge interaction diminishes 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 phosphorous oxygens, relative to that in the S-(aminooethyl)cysteine enzyme. Still, the methyl groups can be accommodated in the vicinity of the phosphorane 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 $>10^3$-fold reduction in $k_{cat}/K_m$.

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

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 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-acetamidinoenzyme and S-(carbamoylmethyl)-cysteine enzymes, the side chains at position 41 are identical except for one of the two heteroatoms attached to the terminal cysteine residue. This value is consistent with other data on the relative strengths of charged and uncharged hydrogen bonds in protein—ligand interactions.23 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)ethylcysteine 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 Materials

BromoethyamineHBr, bromopropylamineHBr, iodoacetamide, and bromoethylthymethylamineHBr were from Sigma Chemical (St. Louis, MO), and were used without further purification. ChloroacetamidineHCl were from Aldrich Chemical (Milwaukee, WI) and were used without further purification.

ChloroacetamidineHCl was synthesized from chloroacetoniitrile and ammonium chloride as described by Schaefer and Peters. Briefly, NaOCH$_3$ was added to a solution of chloroacetoniitrile 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 chloroacetamidineHCl was confirmed by $^1$H NMR (CD$_3$OD; 4.85 ppm relative to tetramethylsilane, $s$, CH$_3$), mass spectrometry (FAB; calculated for C$_5$H$_7$N$_2$O; 102.0413), and melting point determination (92°–94° C; uncorrected). ChloroacetamidineHCl 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 using oligonucleotides AAGGTGTTAACTGGCCCTGATCGATC (for K41R) and AAGGGTTAATTCCGACAGACGATC (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 sulhydryl group in the K41C enzyme was protected from inadvertant air oxidation by reaction with N,N'-dithiodiisocyanic acid (DTNB).

Prior to alkylation of K41C RNase A, the sulhydryl group of Cys41 was deprotected by treating the enzyme with diithiothreitol (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 enzyme 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-acetamidinoenzyme 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 $A_{260}$ M$^{-1}$ cm$^{-1}$ at 250 nm. Steady-state kinetic parameters were determined by fitting initial velocity data to a hyperbolic curve using the program HYPER.30

Acknowledgment. This work was supported by Grant No. GM44783 (NIH). R.T.R. is a Presidential Young Investigator (NSF), Searle Scholar (Chicago Community Trust), and Shaw Scientist (Milwaukee Foundation).