

## A New Remote Subsite in Ribonuclease A\*

(Received for publication, September 28, 1998)

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**The interaction between bovine pancreatic ribonuclease A (RNase A) and its RNA substrate extends beyond the scissile bond. Enzymic subsites interact with the bases and the phosphoryl groups of a bound substrate. We evaluated the four cationic residues closest to known subsites for their abilities to interact with a bound nucleic acid. Lys-37, Arg-39, Arg-85, and Lys-104 were replaced individually by an alanine residue, and the resulting enzymes were assayed as catalysts of poly(cytidylic acid) (poly(C)) cleavage. The values of  $K_m$  and  $k_{cat}/K_m$  for poly(C) cleavage were affected only by replacing Arg-85. Moreover, the contribution of Arg-85 to the binding of the ground state and the transition state was uniform— $K_m$  increased by 15-fold and  $k_{cat}/K_m$  decreased by 10-fold. The contribution of Arg-85 to binding was also apparent in the values of  $K_d$  for complexes with oligonucleotides of different length. This contribution was dependent on salt concentration, as expected from a coulombic interaction between a cationic side chain and an anionic phosphoryl group. Together, these data indicate that Arg-85 interacts with a particular phosphoryl group of a bound nucleic acid. We propose that Arg-85 comprises a new distal subsite in RNase A—the P(–1) subsite.**

The efficiency of enzymatic catalysts is a source of continued interest and inspiration as molecular scientists strive to design new catalysts. A distinguishing characteristic of enzymic catalysts is that they bind to their substrates (1, 2). Binding energy is necessary to compensate for the loss of translational and rotational entropy and for any destabilization of the substrate required to reach the transition state (3, 4). Multivalent contacts between an enzyme and substrate provide much of this required binding energy. Indeed, many enzymes that cleave polymeric substrates have subsites that interact with monomeric units of the substrate.

Bovine pancreatic ribonuclease A (RNase A;<sup>1</sup> EC 3.1.27.5) is a classic model for revealing the physical, chemical, and biological properties of enzymes (5, 6). RNase A is a 13.7-kDa endoribonuclease that binds RNA in a cationic cleft and cleaves on the 3'-side of pyrimidine residues. The cleft contains subsites (B1, B2, and B3) that interact specifically with bases and subsites (P0, P1, and P2) that interact with phosphoryl groups (7, 8). The specificity of RNase A for pyrimidine bases is be-

cause of exclusion of the larger purine bases from the B1 subsite (9). The B2 and B3 subsites prefer to bind purine bases. His-12, His-119, and Lys-41 of the P1 subsite are the residues most central to the catalytic function of the enzyme. The amino acid residues that comprise the P0 (Lys-66) and P2 (Lys-7 and Arg-10) subsites increase the affinity with which the substrate binds to the enzyme and participate indirectly in catalysis (10–12).

Some data portend the existence of additional RNase A binding sites beyond those characterized previously. Three-dimensional structures derived from x-ray diffraction analyses reveal a line of cationic residues stretching well beyond the active site and known subsites (13) (Fig. 1). In addition, cation titration suggests that RNase A can occlude eleven nucleotides of a single-stranded nucleic acid (16) and that binding involves seven coulombic interactions (17).

Here, we search for additional RNase A subsites that interact with bound substrates. We determine the steady-state kinetic parameters for RNA cleavage by the wild-type enzyme and variants in which Lys-37, Arg-39, Arg-85, and Lys-104 are replaced individually by an alanine residue. We also dissect the role of Arg-85 in nucleic acid binding by comparing the affinity of different oligonucleotides for wild-type RNase A and the R85A variant. The results enable us to define a new subsite that interacts with a particular phosphoryl group of a bound nucleic acid.

### EXPERIMENTAL PROCEDURES

**Materials**—All *Escherichia coli* strains, plasmids, enzymes, and reagents were obtained as described elsewhere (11).

**General Methods**—Ultraviolet and visible absorbance measurements were made with a Cary Model 3 spectrophotometer equipped with a Cary temperature controller from Varian (Sugar Land, TX). RNase A concentrations were determined by assuming that  $\epsilon^{0.1\%} = 0.72$  at 277.5 nm (18).

**Rationale for Investigating the Roles of Lys-37, Arg-39, Arg-85, and Lys-104**—An electrostatic surface potential map created from a crystalline structure of wild-type RNase A (Brookhaven National Laboratory Protein Data Bank entry 1RPH, Ref.19) highlights the cationic cleft in the enzyme that interacts with a bound nucleic acid (Fig. 1). The active site of the enzyme comprises His-12, His-119, and Lys-41 (and also known as the P1 subsite) and is located in the center of the cleft. The cationic residues that reside closest to the active site are Lys-7 and Arg-10 (P2 subsite) on one side and Lys-66 (the P0 subsite) on the other side. The roles of Lys-7, Arg-10, and Lys-66 have been characterized in detail (11, 12). Arg-85 and Lys-104 are the next closest cationic residues to Lys-66, and Lys-37 and Arg-39 are the next closest cationic residues to Lys-7 and Arg-10. Hence, we reasoned that one or more of these residues could play a role in catalysis by RNase A. To test this hypothesis, we created variants of RNase A in which Lys-37, Arg-39, Arg-85, and Lys-104 were substituted individually with an alanine residue.

**Site-Directed Mutagenesis**—pBXR is a plasmid that directs the expression of wild-type RNase A in *E. coli* (20). Oligonucleotide-mediated site-directed mutagenesis (21) was performed on single-stranded pBXR isolated from *E. coli* strain CJ236. To produce DNA coding for the K37A, R39A, R85A, and K104A variants, the codon for lysine or arginine was replaced with one for alanine (reverse complement in bold; new restriction endonuclease sites underlined) using oligonucleotides: JG5, CAC-TGGCTTGAACGATCCGCGGTCAGGTTCCG; JG1, CACTGGC TTG-CATGCTTGTGTCAGG; JG4, GGGGTACTTGGAGGATCCGGTCTCC-

\* This work was supported by National Institutes of Health Grant GM-44783 (to R. T. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: RNase A, bovine pancreatic ribonuclease A; A, anisotropy; Fl, fluorescein; MES, 2-(*N*-morpholino)ethanesulfonic acid; poly(C), poly(cytidylic acid);  $T_m$ , temperature at the midpoint of a thermal denaturation curve.

GCGCAGTCGGTG; and JG3, GTTCCCTCGCATGCCACATTGATGTGGCATTTCGCCTG, respectively. Mutagenesis reactions were transformed into competent DH5 $\alpha$  cells, and cDNA sequences from isolated transformants were determined with an ABI 373 automated sequencer.

**Protein Production and Purification**—Wild-type RNase A and the K37A, R39A, R85A, and K104A variants were produced in *E. coli* strain BL21(DE3) as described elsewhere (11). Peak symmetry on chromatography elution profiles, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and  $A_{280}/A_{260}$  ratios greater than 1.8 indicated that the proteins were >99% pure. Purified proteins were dialyzed exhaustively against H<sub>2</sub>O, lyophilized, and stored at -70 °C.

**Thermal Denaturation**—Stabilities of the wild-type and variant proteins were determined by thermal denaturation studies in 0.10 M MES-NaOH buffer, pH 6.0, as described elsewhere (22).

**Steady-state Kinetic Analysis**—Spectrophotometric assays were used to determine steady-state kinetic parameters for the cleavage of poly(C). The  $\Delta\epsilon$  for this reaction is 2380 M<sup>-1</sup> cm<sup>-1</sup> at 250 nm (9). Concentrations of poly(C) were determined by assuming that  $\epsilon = 6200$  M<sup>-1</sup> cm<sup>-1</sup> per nucleotide at 268 nm (23). Assays were performed at 25 °C in 0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M), poly(C) (30  $\mu$ M - 1.6 mM), and enzyme (0.75 pM - 3.1 nM). Values of  $k_{\text{cat}}$ ,  $K_M$ , and  $k_{\text{cat}}/K_M$  were determined from initial velocity data with the program HYPERO (24).

**Fluorescence Anisotropy**—Fluorescence anisotropy assays were used to quantitate the binding of single-stranded DNA to wild-type RNase A and the R85A variant. DNA is a useful analog of RNA because RNase A binds to but does not cleave DNA (25). Fluorescein (Fl), incorporated during the final coupling step of DNA synthesis, was attached to the 5'-end of two oligonucleotides via a six-carbon spacer. These oligonucleotides, Fl~d(AUAA) and Fl~d(UAA), were designed to test for the existence of an additional phosphoryl group subsite on the 5'-side of the scissile bond (Scheme 1).

Fluorescein-labeled oligonucleotides were obtained in purified, desalted form from Promega. The oligonucleotide concentrations were determined by assuming that  $\epsilon = 66250$  M<sup>-1</sup> cm<sup>-1</sup> at 260 nm for Fl~d(AUAA) and  $\epsilon = 60300$  M<sup>-1</sup> cm<sup>-1</sup> at 260 nm for Fl~d(UAA) (26).

Fluorescence anisotropy was measured at room temperature (23  $\pm$  2 °C) in 0.020 M MES-NaOH buffer, pH 6.0, containing NaCl (0.025 M) and oligonucleotide (2.5 nM) on a Beacon Fluorescence Polarization System (Panvera, Madison, WI) as described elsewhere (11). Data were fitted to Eq. 5 of Ref. 11 to obtain values of  $K_d$ .

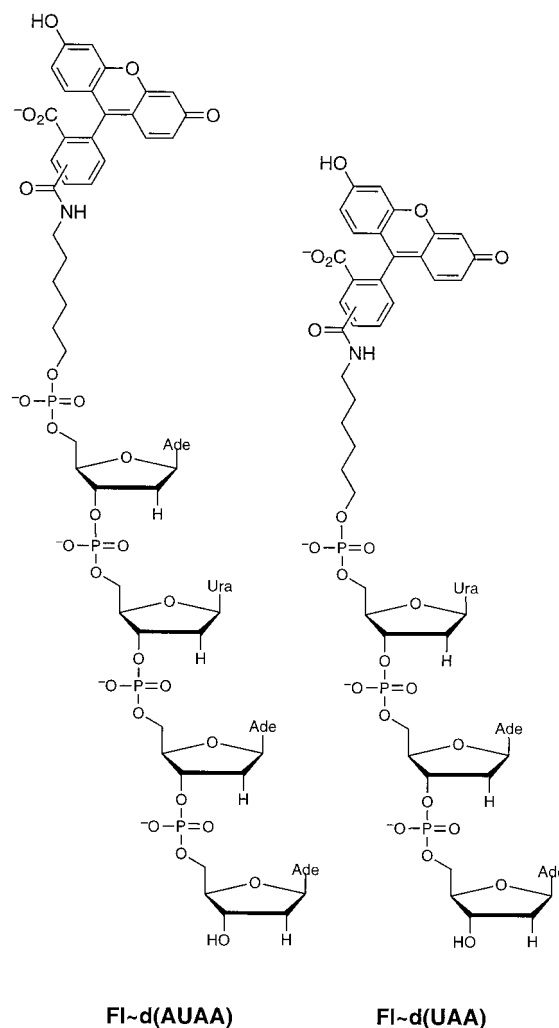
## RESULTS

**Catalysis of Poly(C) Cleavage by RNase A Variants**—Steady-state kinetic parameters for the cleavage of poly(C) by wild-type RNase A and the K37A, R39A, R85A, and K104A variants are listed in Table I. Also listed in this table are the values of  $T_m$  for the five proteins. These values indicate that the kinetic parameters determined at 25 °C are indeed those of the native proteins.

Of the four residues investigated in this study, Arg-85 has the most dramatic effect on the kinetic parameters for the cleavage of poly(C). The  $k_{\text{cat}}$  value is similar to that of the wild-type enzyme, but the  $K_M$  value differs dramatically, being >15-fold higher than that for the wild-type enzyme. The value of  $k_{\text{cat}}/K_M$  for poly(C) cleavage by R85A RNase A is 10-fold lower than that for the wild-type enzyme.

Replacing Lys-37, Arg-39, or Lys-104 with alanine has little change on the kinetic parameters for cleavage of poly(C). The  $k_{\text{cat}}$ ,  $K_M$ , and  $k_{\text{cat}}/K_M$  values for poly(C) cleavage by K37A RNase A and R39A RNase A are virtually indistinguishable from those of wild-type RNase A. The  $k_{\text{cat}}$  and  $K_M$  values for poly(C) cleavage by K104A RNase A are both 2-fold lower than those for the wild-type enzyme, and the  $k_{\text{cat}}/K_M$  value is similar.

**Oligonucleotide Binding to Wild-Type RNase A and the R85A Variant**—The specific interaction between Arg-85 and single-stranded DNA was evaluated by fluorescence anisotropy assays. These assays employed two fluorescein-labeled DNA oligonucleotides that differ only by an adenosine 5'-phosphate. The ligands, Fl~d(UAA) and Fl~d(AUAA), were modeled after d(ATAAG), which forms a complex with known three-dimen-



SCHEME 1

TABLE I

Steady-state kinetic parameters for the cleavage of poly(cytidylic acid) by wild-type ribonuclease A<sup>a</sup>, K37A ribonuclease A, R39A ribonuclease A, R85A ribonuclease A, and K104A ribonuclease A<sup>b</sup>

Ribonuclease A ( $T_m$ ) <sup>c</sup>	$k_{\text{cat}}$	$K_M$	$k_{\text{cat}}/K_M$
° C	s <sup>-1</sup>	mM	10 <sup>6</sup> M <sup>-1</sup>
Wild Type (62)	507 $\pm$ 15	0.089 $\pm$ 0.009	5.7 $\pm$ 0.5
K37A (61)	539 $\pm$ 10	0.071 $\pm$ 0.004	7.6 $\pm$ 0.4
R39A (62)	541 $\pm$ 28	0.096 $\pm$ 0.002	5.7 $\pm$ 1.1
R85A (62)	659 $\pm$ 80	1.42 $\pm$ 0.30	0.46 $\pm$ 0.04
K104A (62)	281 $\pm$ 5	0.042 $\pm$ 0.004	6.4 $\pm$ 0.5

<sup>a</sup> Data from Ref. 11.

<sup>b</sup> Data were obtained at 25 °C in 0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M), enzyme (0.75–3.1 nM), and poly(C) (30  $\mu$ M–1.6 mM).

<sup>c</sup> Values of  $T_m$  are reported  $\pm$  2 °C.

sional structure (PDB entry 1RCN; Ref. 15). The single pyrimidine in d(ATAAG) (and presumably in Fl~d(UAA) and Fl~d(AUAA)) binds to RNase A specifically in the B1 subsite.

Data for the binding of Fl~d(UAA) and Fl~d(AUAA) to wild-type RNase A is shown in Fig. 2A. Data for the binding of Fl~d(UAA) and Fl~d(AUAA) to R85A RNase A is shown in Fig. 2B. Although the anisotropy values of the bound ( $A_{\text{max}}$ ) and unbound oligomer ( $A_{\text{min}}$ ) varied slightly between experiments, the total change in anisotropy ( $\Delta A$ ) remained constant for all experiments. This value ( $\Delta A = 120$  mA) is consistent with that seen previously for the binding of Fl~d(AUAA) (11)

and Fl~d(UAA) (27) to wild-type RNase A.

As shown in Fig. 2, Fl~d(AUAA) binds to wild-type RNase A to form a complex with a  $K_d$  value of  $3.0 \mu\text{M}$ . This value is 8-fold lower than that for the complex of Fl~d(UAA) and wild-type RNase A. This result suggests that there are specific interactions between RNase A and the 5'-phosphoryl group or adenine base of the bound nucleic acid. Fl~d(AUAA) binds to the R85A variant to form a complex with a  $K_d$  value of  $9.6 \mu\text{M}$ . This value is 2.4-fold lower than the wild-type RNase A~Fl~d(UAA) complex, again suggesting that RNase A interacts specifically with the additional adenosine 5'-phosphate. Fl~d(UAA) binds to wild-type RNase A and the R85A variant with similar affinity.

#### DISCUSSION

The active site and several other subsites of RNase A have been well characterized (5, 6). The goal of this work is to identify additional subsites in RNase A that interact with the RNA substrate during catalysis. Using both kinetic and thermodynamic assays, we find that the side chain of Arg-85 interacts with the phosphoryl group of a bound substrate. In addition, we show that Lys-37, Arg-39, and Lys-104 do not, at least individually, comprise RNase A subsites.

Thirty years ago, Takahashi (1968) proposed roles for Arg-39 and Arg-85 based on a loss of ribonucleolytic activity upon covalent modification of these residues (28). Subsequent affinity labeling and molecular modeling studies led Cuchillo and co-workers (29) to predict that Lys-104 and Lys-37 comprise an additional phosphoryl group subsite on the 5'- and 3'-side, respectively, of the scissile bond. After determining the crystalline structure of the RNase A~d(ATAAG) complex, Cuchillo and co-workers (15) included Arg-85 as a possible subsite residue. The oligonucleotide in the crystalline complex lacked a 5'-phosphoryl group, and no interaction between Arg-85 or Lys-104 and d(ATAAG) was apparent. Likewise, disorder in the crystalline complex precluded detection of new interactions on the 3'-side of the P2 subsite. Still, an electrostatic potential map of the RNase A surface concurred with prior speculations—Lys-37, Arg-39, Arg-85, and Lys-104 were good candidates for additional enzymic subsites (Fig. 1).

**Lys-37 and Arg-39 in Catalysis**—Replacing Lys-37 or Arg-39 with an alanine residue has little effect on the kinetic parameters for the cleavage of poly(C). As listed in Table I,  $k_{\text{cat}}$ ,  $K_m$ , and  $k_{\text{cat}}/K_m$  are all within 2-fold of the values for wild-type RNase A. Despite the proximity of Lys-37 and Arg-39 to the P2 subsite and their cationic side chains, which could interact with a phosphoryl group of RNA, these data suggest that Lys-37 and Arg-39 do not contribute to catalysis. Lys-37 is not conserved by evolution. Of the 41 pancreatic ribonucleases of known sequence, only those from bovine, eland, rat, and guinea pig have a lysine residue at position 37 (30) (glutamine is most prevalent at this position). Likewise, Arg-39 is not conserved. Of the 41 pancreatic ribonucleases of known sequence, only 30 have an arginine residue at position 39 (30).

**Lys-104 in Catalysis**—Replacing Lys-104 with an alanine residue has a small effect on catalysis. Although the value of both  $k_{\text{cat}}$  and  $K_m$  for K104A RNase A are 2-fold lower than those of the wild-type enzyme, the change to the value of  $k_{\text{cat}}/K_m$  is not significant. These results suggest that Lys-104 has little importance in the RNase A-catalyzed cleavage of RNA. The similarity in the kinetic parameters for poly(C) cleavage by K104A RNase A and the wild-type enzyme was not anticipated from a consideration of phylogenetics. In contrast to Lys-37 and Arg-39, Lys-104 is conserved highly. The side chain of residue 104 is cationic in 39 of 40 pancreatic ribonucleases whose sequences have been determined, with all but four of these residues being lysine (30).

**Arg-85 in Catalysis**—Residue 85 is the most conserved resi-

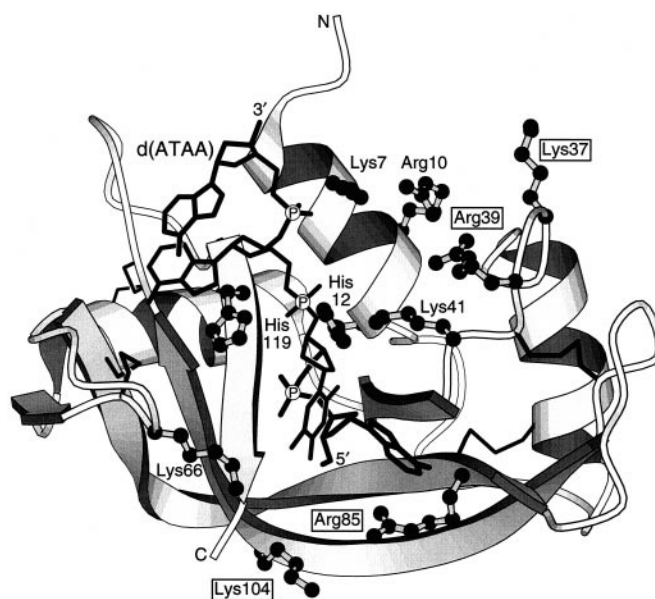


FIG. 1. Three-dimensional structure of the ribonuclease A~d(ATAAG) complex. Residues in the P0 (Lys-66), P1 (His-12/Lys-41/His-119), and P2 (Lys-7/Arg-10) subsites are shown, along with Lys-37, Arg-39, Arg-85, and Lys-104 (examined herein). This map was created with the program MOLSCRIPT Ver. 1.2 (14) using coordinates determined by x-ray diffraction analysis (PDB entry 1rcn; Ref. 15). Electron density was not apparent for the guanosine 5'-phosphate.

due of the four investigated herein. An arginine is present at position 85 in 40 of 41 pancreatic ribonucleases (30). The single exception is mouse pancreatic ribonuclease, which has a histidine at this position. Based on its conservation by evolution and its proximity to the active site, Arg-85 appeared likely to play a role in substrate binding.

The impaired kinetic parameters for the cleavage of poly(C) by R85A RNase A suggest a significant role for residue 85. Although the value of  $k_{\text{cat}}$  for poly(C) cleavage remains essentially unchanged, those of  $K_m$  and  $k_{\text{cat}}/K_m$  differ dramatically (Table I). Interestingly, the >15-fold increase in the value of  $K_m$  is larger than that for K41A RNase A, which has the largest  $K_m$  value observed previously for an RNase A variant.<sup>2</sup> In addition, the value of  $k_{\text{cat}}/K_m$  for poly(C) cleavage by R85A RNase A is 10-fold lower than that of the wild-type enzyme. Thus, Arg-85 makes a uniform contribution toward binding the ground state and the rate-limiting transition state during catalysis.

**Arg-85 in Binding**—Coulombic interactions can form between the cationic side chains of a protein and the anionic phosphoryl groups of a nucleic acid. The driving force for complex formation is the release of cations from the nucleic acid upon complex formation (17). Accordingly, proteins often bind more strongly to nucleic acids in solutions that have a lower concentration of cations. Indeed, the affinity of Fl~d(AUAA) for RNase A increases with decreasing sodium concentration (11). The  $K_d$  values for the RNase A~Fl~d(AUAA) and RNase A~Fl~d(UAA) complexes in the solution used in the poly(C) cleavage assays (0.10 M MES-NaOH, pH 6.0, containing NaCl (0.10 M)) are  $88 \mu\text{M}$  (11) and  $130 \mu\text{M}$  (27), respectively. These  $K_d$  values differ by only 1.5-fold. Yet, in the low salt concentration solution used in our binding experiments (0.020 M MES-NaOH buffer, pH 6.0, containing NaCl (0.025 M)), the  $K_d$  values for the RNase A~Fl~d(AUAA) and RNase A~Fl~d(UAA) complexes are 3.0 and  $24 \mu\text{M}$ , respectively, differing by 8-fold (Figs. 2 and 3). These salt-concentration effects are consistent with the exist-

<sup>2</sup> J. M. Messmore and R. T. Raines, unpublished results.

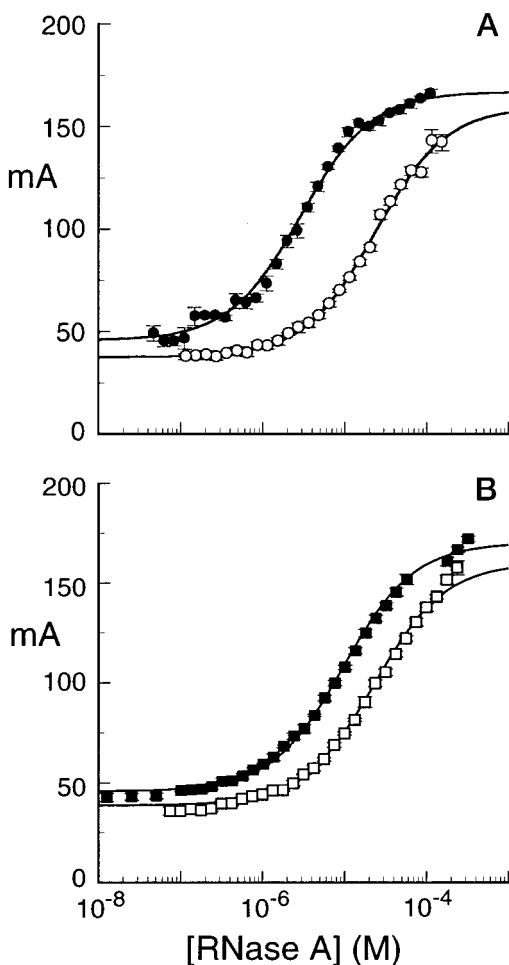


FIG. 2. **Binding of ribonucleases to oligonucleotides.** A, wild-type ribonuclease A to Fl~d(AUAA) (●;  $K_d = 3.0 \mu\text{M}$ ) and Fl~d(UAA) (○;  $K_d = 24 \mu\text{M}$ ). B, R85A ribonuclease A to Fl~d(AUAA) (■;  $K_d = 9.6 \mu\text{M}$ ) and Fl~d(UAA) (□;  $K_d = 23 \mu\text{M}$ ). Binding was assayed at  $23 \pm 2^\circ\text{C}$  by fluorescence anisotropy in 0.020 M MES-NaOH buffer, pH 6.0, containing NaCl (0.025 M) and oligonucleotide (2.5 nM). Each data point is the average of six to eight measurements, with the standard deviation for each point indicated by the bars. Curves are best fits to Eq. 5 of Ref. 11.

ence of a coulombic interaction between the cationic side chain of Arg-85 and the additional anionic phosphoryl group of Fl~d(AUAA).

Why is the affinity of R85A RNase A for Fl~d(AUAA) greater than that for Fl~d(UAA)? In the crystalline RNase A-d(ATAAG) complex (15), the side chain of Arg-85 is directed toward the 5'-hydroxyl group of d(ATAAG), which is in the position that would be occupied by the 5'-phosphoryl group of Fl~d(AUAA). This interaction is removed upon replacing Arg-85 with alanine. Still,  $C^\beta$  and  $C^\gamma$  of Pro-42 make van der Waals contact with the 5'-adenine base of d(ATAAG). This interaction could be responsible for the additional binding energy upon complex formation with the longer oligonucleotide.

**Implications for Catalysis**—The cationic residues of RNase A that are known to interact with RNA are distributed evenly about the active site (Fig. 4). Arg-85 and Lys-66 are on the 5'-side, and Lys-7 and Arg-10 (P2) are on the 3'-side. In contrast to the substrate, the products of RNA cleavage each interact with only two of these four residues. This symmetric disposition of the phosphoryl group binding subsites is thus optimal for maximizing the binding of substrate while minimizing the binding of products.

**Conclusions**—RNase A has known subsites that enable the

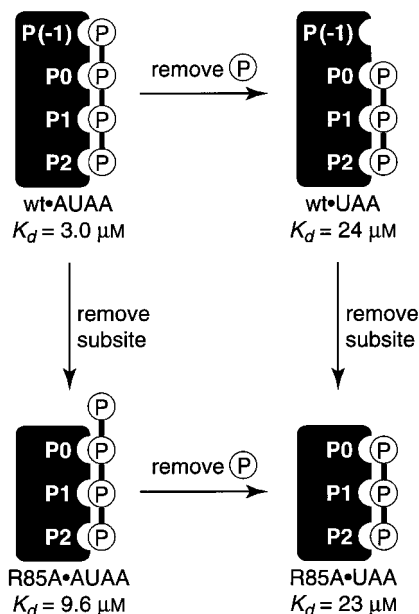


FIG. 3. **Effect of removing an enzymic subsite, a nucleoside 5'-phosphate, or both on the affinity of ribonuclease A for a nucleic acid.** Values of  $K_d$  are from the data in Fig. 2.

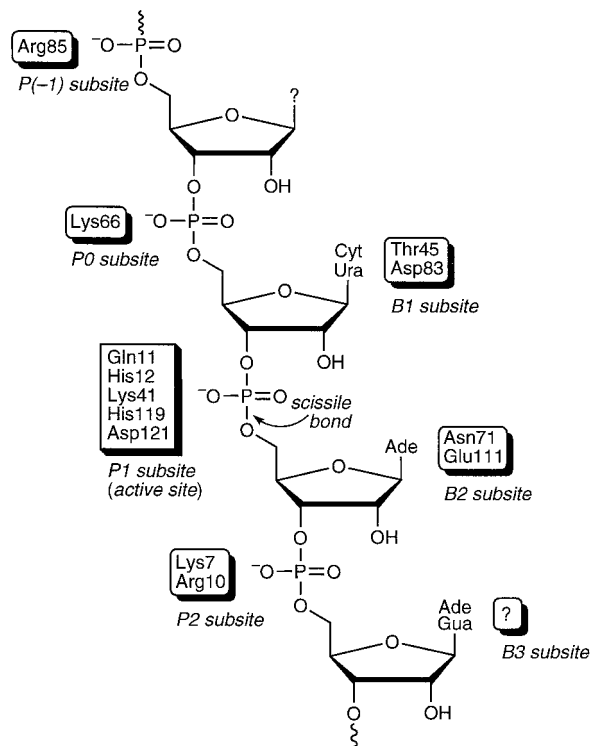


FIG. 4. **Ribonuclease A-RNA interactions.** The scissile bond is indicated. B and P refer to base and phosphoryl group binding sites, respectively. The thirteen indicated enzymic residues have been shown by site-directed mutagenesis to make a contribution to substrate binding or turnover (or both) (6). Arg-85 is shown herein to comprise the P(-1) subsite.

multivalent interaction with the bases (B1, B2, and B3) and phosphoryl groups (P0, P1, and P2) of an RNA substrate. The results of kinetic and thermodynamic experiments indicate that Arg-85 has an important role in nucleic acid binding by RNase A. Based on the proximity of Arg-85 to the P0 subsite, the dramatic change in the  $K_m$  value for poly(C) cleavage, and the differential binding affinities of Fl~d(AUAA) and Fl~d(UAA) for the wild-type protein and R85A variant, we

conclude that the side chain of Arg-85 constitutes a new enzymic subsite, which we call "P(-1)" (Fig. 4).

*Acknowledgments*—We are grateful to B. R. Kelemen and Dr. L. W. Schultz for helpful discussions, and to T. E. Singleton, V. A. Munsen, and M. M. Steiniger-White for assistance with preparation and purification of the variant proteins.

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