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Role of Phe120 in the Activity and Structure of Bovine Pancreatic Ribonuclease A[1]

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Phenylalanine120 is a candidate residue juxtaposing catalytic His12 and His119 in ribonuclease A (RNase A). To clarify its role in construction of the catalytic center, Phe120 was replaced by alanine, tryptophan, leucine, or glutamic acid by site-directed mutagenesis. The transphosphorylation and hydrolysis activities of the mutant RNase As, respectively, toward cytidinyl 3′,5′ adenosine (CpA) and cytidine 2′,3′ cyclic monophosphate (C>p) were compared with those of the wild type enzyme. The K_m values of the two reactions increased markedly with slight changes in the k_{cat} values. The pK_e values of His12 and His119 in the wild type and mutant enzymes, estimated from the pH dependence of the k_{cat}/K_m values, showed little change. The rate of carboxymethylation was reduced markedly by the mutations. The K_i values of the phosphate anion as to hydrolysis activity increased only slightly when Phe120 was replaced by leucine, tryptophan, or alanine. These findings suggest that Phe120 participates in the binding of the substrate, juxtaposing His12 and His119, and in stabilizing the transition state intermediate in the hydrolysis reaction. Furthermore, the decreases in the thermal denaturation temperatures of all the mutants, particularly F120E, indicate that Phe120 also helps maintain the conformational stability of RNase A.

Key words: carboxymethylation of histidine, catalytic histidine residues, catalytic role of phenylalanine, RNase A.

Bovine pancreatic ribonuclease A (RNase A) [EC 3.1.27.5] is a pyrimidine base-specific endoribonuclease which cleaves the 3′,5′-phosphodiester bonds of single strand RNA when the base of the nucleotide at the 3′ position is a pyrimidine. The structure and function relationship of RNase A has been studied extensively by means of classical methods, including chemical modification (*1-4*). Studies involving site-directed mutagenesis focused on the roles of individual amino acid residues, especially neutral amino acid residues, in structural maintenance (*5*), the folding pathway (*6, 7*), and the catalytic function (*8-10*) have been performed because of the successful establishment of expression systems in *Escherichia coli* (*5, 11-14*).

Certain aromatic residues in the active sites of ribonucleases were reported to participate in the catalytic activity. In RNase A, Tyr97, positioned near the catalytic residue, Lys41, has been shown to be an important residue determining the orientation of Lys41, thereby affecting the catalytic activity (*15*). In RNase T1, Phe100 affects the catalytic efficiency by promoting the polarization of catalytic residues Glu58 and His92, and by accelerating the dissociation of products (*16*).

Phe120, a conserved residue in mammalian pancreatic ribonucleases (*4*), is positioned near catalytic His12 and His119 in the three dimensional structure of RNase A (*3*), and seems to form a pyrimidine base-binding pocket with Thr45 and Ser123 (*17*). Phe120 also may function in catalysis; *e.g.*, the chemical substitution of leucine, isoleucine, or tryptophan for Phe120 in peptide 111-124 significantly altered the catalytic activity when the peptide was mixed with RNase 1-118 (*18, 19*). X-ray analysis of a mixture of RNase 1-118 and peptide 111-124, in which Phe120 was replaced by leucine or tyrosine, showed no marked structural changes that would explain the decrease in catalytic activity (*20*). We have examined in detail the role of Phe120 in the structure and catalysis of RNase A using site-directed mutagenesis.

MATERIALS AND METHODS

Materials—Plasmid pSJII165 and *E. coli* strain HMS-174(DE3)-pLys(S) were gifts from Dr. H.A. Scheraga (Cornell University, USA). *E. coli* strains JM109 and BMH71-18mutS were used as the host cells for cloning and mutagenesis.

Mutagenesis—Mutant plasmids were constructed by the Deng and Nickoloff method using a double stranded plasmid pSJII165 and two kinds of oligonucleotide primers (*21*). The selection primer designed to replace a unique restric-

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Abbreviations: CpA, citydinyl 3′,5′ adenosine; C>p, cytidine 2′,3′ cyclic monophosphate; RNase A, ribonuclease A.

tion site from *Sma*I to *Pvu*II was 5′-ATT AAT GCA GCA GCT GAG ATC TCG A-3′. The target primers designed to replace the phenylalanine codon with another amino acid codon (alanine, tryptophan, leucine, or glutamic acid) were 5′-C GTT CCT GTC CAC GCT (for Ala)/TGG (for Trp)/ TTG (for Leu)/GAG (for Glu) ATC GAC GCC AGT GTT T-3′.

All the mutations introduced into pSJII165 were confirmed by DNA sequencing with an Applied Biosystems 373A DNA sequencer (Perkin Elmer) using the dideoxy terminator sequencing method.

Protein Expression and Purification—RNase A was expressed as a gene10 fusion protein by using the expression system of Scheraga *et al.* (*5*). Enzyme purification and reconstruction were performed according to the published procedures (*5*) with the following minor modifications: a Bio Scale S cation-exchange column (Bio Rad, 7×52 mm) was used to purify the sulfonated enzymes eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM formic acid buffer, pH 4.0, containing 3 M urea. Regeneration of the wild type RNase A was monitored by measuring the activity toward C>p, and that of mutant RNase A was monitored by measuring the activity toward CpA. The reconstructed RNase A solution was loaded onto a Bio Scale S column equilibrated with 25 mM sodium phosphate buffer, pH 6.5, which was then eluted with a linear gradient of 0 to 0.3 M NaCl. For the wild type RNase A, three active peaks were eluted with this gradient. The last peak, exhibiting the highest specific activity, was collected as the recombinant RNase A. After exchanging the phosphate buffer for distilled water, the recombinant RNase A solution was concentrated and kept at 4°C.

Determination of Kinetic Parameters—The kinetic parameters of the transphosphorylation reaction for CpA and the hydrolysis reaction for C>p, respectively, were determined by HPLC and spectrophotometry (*9*). Transphosphorylation activity was measured with CpA concentrations of 0.2 to 28 mM as follows: After incubation of a 90 μl reaction mixture containing CpA for 5 min at 25°C, 10 μl of an RNase A solution was added and then the whole mixture incubated for 2 min at 25°C. A 100 μl portion of 8 M guanidine-HCl then was added to stop the reaction. Twenty to 50 μl of the mixture was loaded onto an ODS column (Cosmosil 5C18AR, 46×150 mm) equilibrated with 10 mM sodium phosphate buffer, pH 3.0. The reaction product, C>p, was eluted isocratically and detected by monitoring at A_{265}. Hydrolytic activity was measured by recording the increase in absorbance at 296 nm ($\Delta\varepsilon_{296}$ = 516.4 M^{-1} cm^{-1}) with C>p concentrations from 0.3 to 13 mM. Inhibition of the hydrolytic activity by the phosphate anion was measured as above, but in the presence of 0 to 25 mM phosphate and with two different C>p concentrations. All the kinetic measurements were made in 0.2 M sodium acetate buffer, pH 5.5, at 25°C.

pH Dependence of k_{cat}/K_m—The transphosphorylation and hydrolysis activities were measured with substrate concentrations of about 1/5 the K_m values. The k_{cat}/K_m values were estimated using the equation,

$$v = k_{cat}/K_m[E][S] \quad (1)$$

Sodium acetate, MES, HEPES, and Tris-HCl buffers were used, respectively, for pH values between 4.0 and 5.9, 5.3 and 6.6, 6.9 and 7.3, and 6.9 and 8.1, respectively. The CpA concentrations used were 87 μM for the wild type enzyme, 0.44 mM for F120L, 0.87 mM for F120W, and 1.8 mM for F120A and F120E. The C>p concentrations used were 0.11 mM for the wild type enzyme, 0.28 mM for F120L, 0.70 mM for F120W, 0.56 mM for F120A, and 0.97 mM for F120E. The extinction coefficients at each pH were determined by obtaining the spectra of C>p and 3′-CMP. The pH dependence of the extinction coefficients for 3′CMP and C>p, pre-determined at 286 and 290 nm, was used for the calculations. The hydrolytic activity toward C>p was measured spectrophotometrically by recording the increase in absorbance at 286 nm for the wild type enzyme, and F120L and F120A, and that at 290 nm for F120W and F120E.

Chemical Modification with Iodoacetate—Carboxymethylation was performed by the method of Crestfield *et al.* (*2*) with the modification that each recombinant RNase A was incubated at 25°C with iodoacetate in a 1:1,000 molar ratio. Residual activity for CpA as the substrate was also measured. An attempt was made to determine the amount of mutant RNase A bearing 1-carboxymethyl His119 or 3-carboxymethyl His12 with a Bio Scale S column and elution with a linear phosphate ion gradient of 50 to 500 mM.

Circular Dichroism Spectroscopy—CD spectra from 195 to 250 nm were recorded at room temperature with a Jasco J720 spectropolarimeter at the enzyme concentration of 12 μM in 10 mM MES buffer, pH 6.0, in a cell with a 0.1-cm-long optical path.

Thermal Denaturation—Thermal denaturation was monitored as the change in the [θ] value at 222 nm as a function of temperature. The temperature was increased 0.5°C per minute. A water-jacketed cell with a 1-cm-long optical path was used to control the temperature. A thermometer placed inside the cell compartment was used to record the thermal denaturation temperature. The enzyme concentration was adjusted to 1.5 μM with 10 mM MES buffer, pH 6.0, containing 0.1 M KCl.

Protein Concentration—Protein concentrations were determined by the bicinchoninic acid method (*22*) (Pierce, Rockford, Illinois) for the crude extract and sulfonated RNase A, and by amino acid analysis for the purified protein.

RESULTS

Expression and Purification of the Wild Type and Mutant RNase As—About 100 to 300 mg of a fusion protein was obtained from 1 liter of culture. After the final purification step, the wild type and mutant RNase As each gave a single band on SDS-PAGE that was identical with that of authentic bovine pancreatic RNase A. The yields after the purification steps are shown in Table I. Both the wild type and mutant enzymes showed nearly identical

TABLE I. **Expression and purification of the wild type and mutant RNase As, per liter culture.**

Purification step	Yield (mg)				
	Wild type	F120L	F120W	F120A	F120E
Expressed fusion protein	150	280	100	200	290
Products of factor Xa *digestion*	15	10	7.0	16	14
Sulfonated RNase A	4.2	2.8	1.1	3.5	1.8
Folded RNase A	1.2	0.6	0.2	0.9	0.4

overall yields throughout the purification procedures.

Refolding of the Wild Type and Mutant RNase As from the Sulfonated Form—Although the yields from the sulfonated to mature forms were almost the same for all the recombinant RNase As (20-30%), the folding rates differed markedly for the wild type and the two mutant enzymes, F120A and F120E (Fig. 1). The activity of F120E did not reach the maximum level even after 50 h incubation, at which time the activities of the wild type and other mutant enzymes had already reached their maximums.

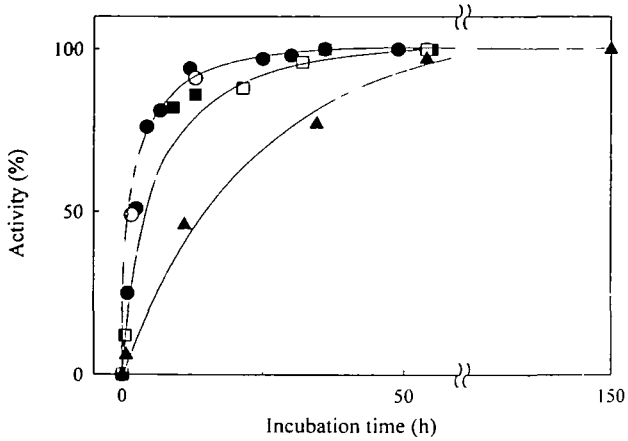

Fig. 1. **Refolding of the wild type and mutant RNase As.** Desalted sulfonated recombinant RNase A was incubated with oxidized and reduced glutathione mixtures for the indicated periods. The transphosphorylation activity (●, wild type; ○, F120L; ■, F120W; □, F120A; ▲, F120E) toward CpA was measured at various times.

Kinetic Parameters—The kinetic parameters are given in Table II. The mutant enzymes exhibited marked levels of catalytic activity for both CpA and C>p, the respective K_m values for CpA and C>p increasing 6 to 20 times and 3 to 13 times. The increases in K_m for CpA were about twice those for C>p in the mutants. The k_{cat} values of the mutants for C>p decreased 1.4- to 10-fold. Whether the k_{cat} values for CpA increased or decreased depended on the amino acid residue introduced at Phe120 position; a 1.6-fold increase for F120L, no change for F120A, and a 3-fold decrease for both F120W and F120E.

The hydrolytic activities toward C>p of the wild type and all the mutant RNase As were competitively inhibited by the phosphate anion. F120L, F120W, and F120A exhibited 2-fold increases in their K_i values, whereas F120E

TABLE III. **K_i values of the phosphate anion as to the hydrolysis of C>p by the wild-type and mutant RNase As.**

Enzyme	K_i (mM)
Wild type	6.4±0.8
F120L	10 ±2
F120W	11 ±1
F120A	10 ±1
F120E	4.8±0.1

K_i values are the average values for 3 measurements.

TABLE II. **Kinetic parameters of the wild-type and mutant RNase As.**

Enzyme	CpA			C>p			$k_{cat}^{CpA}/k_{cat}^{C>p}$
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}\times10^3$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	
Wild type	0.67±0.05	1,800±100	2,700±200	0.62±0.06	2.7 ±0.2	4,200±300	670
F120L	3.5 ±0.4	2,800±200	800± 30	1.7 ±0.1	1.8 ±0.2	1,100±100	1,600
F120W	7.3 ±0.9	470± 60	64± 2	4.0 ±0.2	0.23±0.02	60± 3	2,000
F120A	13 ±3	1,300±200	96± 10	7.9 ±0.4	0.62±0.03	77± 1	2,100
F120E	12 ±1	590± 50	49± 4	5.5 ±0.3	0.32±0.02	56± 1	1,800

All parameter values are the average values for 3 measurements.

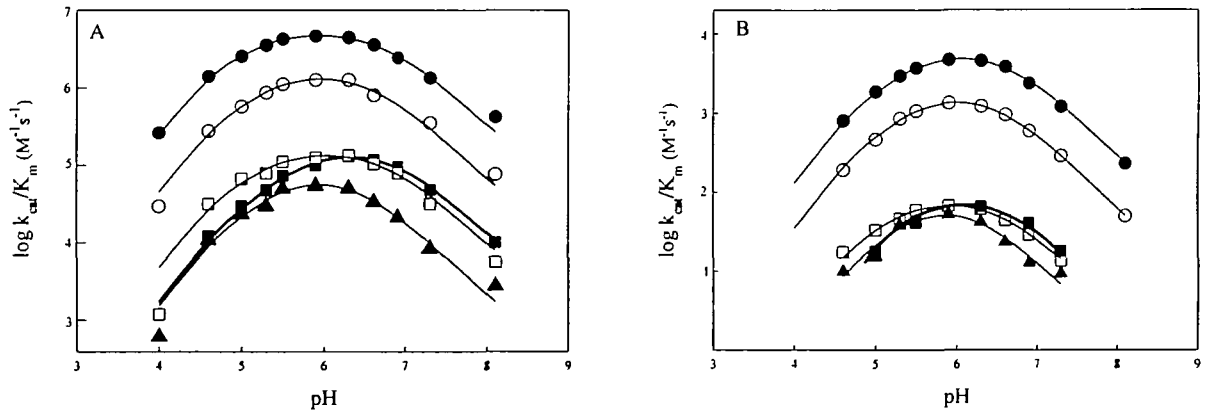

Fig. 2. **pH-dependence of k_{cat}/K_m for the transphosphorylation (A) and hydrolysis (B) reactions of the wild type and mutant RNase As.** CpA (●, 87 μM for wild type RNase A; ○, 0.44 mM for F120L; ■, 0.87 mM for F120W; □, 1.8 mM for F120A; ▲, 1.8 mM for F120E) and C>p (●, 0.11 mM for wild type RNase A; ○, 0.28 mM for F120L; ■, 0.4 mM for F120W; □, 0.56 mM for F120A; ▲, 0.97 mM for F120E) were used. The pK_{e1} and pK_{e2} values for the transphosphorylation reaction were 5.0 and 6.8 for the wild type enzyme, 5.3 and 6.5 for F120L, 5.8 and 6.8 for F120W, 5.3 and 6.7 for F120A, and 5.6 and 6.3 for F120E. The pK_{e1} and pK_{e2} values for the hydrolysis reaction were 5.5 and 6.6 for the wild type enzyme, 5.6 and 6.4 for F120L, 5.8 and 6.6 for F120W, 5.3 and 6.5 for F120A, and 5.8 and 5.9 for F120E. The standard deviation of the pK_e values of wild type enzyme for both reactions was about 0.1 per pH unit.

only showed a slight decrease (Table III).

pH Dependence of k_{cat}/K_m Values—The pK_e values of His12 and His119 could be estimated as pK_e values by measuring the pH dependence of k_s/K_s (*23, 24*). According to Eftink and Biltonen (*24*), the pH dependence of k_s/K_s for a substrate containing a cytosine base can be fitted with the equations;

$$EH_2 \overset{K_{e1}}{\rightleftharpoons} EH \overset{K_{e2}}{\rightleftharpoons} E \quad (2)$$

$$k_s/K_s = k_c/\{K_c(1+[H^+]/K_{e1}+K_{e2}/[H^+]+K_{e2}/K_{e1}) \times(1+[H^+]/K')\} \quad (3)$$

where $[H^+]$ is the hydrogen ion concentration, and k_c and K_c, respectively, are the pH-independent rate and Michaelis constants. K_{e1} and K_{e2} are the apparent acid dissociation constants for two catalytic residues. The term, $1+[H^+]/K'$, in Eq. 3 was calculated by taking the pK of the 3-imino group of the cytosine base as 4.05. Here, k_{cat}/K_m values have been used in place of k_s/K_s ones.

The pH dependence of the k_{cat}/K_m values of the transphosphorylation and hydrolysis activities of the wild type and mutant RNase As is shown in Fig. 2, from which pK_{e1} and pK_{e2} were calculated according to Eqs. 2 and 3. The wild type enzyme exhibits $pK_{e1}=5.1$ and $pK_{e2}=6.8$ for the transphosphorylation reaction against CpA, which differ somewhat from the values of Rosario and Hammes ($pK_{e1}=5.4$ and $pK_{e2}=6.4$), whereas the pK_e values for the hydrolysis reaction ($pK_{e1}=5.5$ and $pK_{e2}=6.6$) are almost identical to their values (*23*). For F120L and F120A, the pK_{e1} and pK_{e2} values are almost identical to the values for the wild type enzyme. In contrast, the pK_{e1} values of F120W and F120E are higher, and the pK_{e2} values of F120E lower.

Iodoacetate Modification of Two Catalytic Histidine Residues—Marked reductions in the transphosphorylation activities of the wild type and mutant enzymes occurred when they were subjected to carboxymethylation (Fig. 3). The inactivation rates for the mutant enzymes differed from the rate for the wild type: The pseudo first order rate constant of inhibition was 1.4×10^{-4} s^{-1} for the wild type enzyme, 2.8×10^{-5} s^{-1} for F120L, F120A, and F120E, and 1.1×10^{-5} s^{-1} for F120W.

Our attempt to separate the 1-carboxymethyl His119 modified enzyme species from the unmodified mutant enzymes on a Bio Scale S column was unsuccessful, but the wild type enzyme was separated. It is impossible, therefore, to give the ratios of the two products, 1-carboxymethyl His119 *vs.* 3-carboxymethyl His12 RNase A, for the mutant enzymes.

Secondary Structures of the Wild Type and Mutant RNase As—The CD spectra of the wild type, F120L, and F120A were nearly identical, whereas the spectra of F120W and F120E differed slightly from the spectrum of the wild type (Fig. 4). Tryptophan and glutamic acid residues might disturb the secondary structure, but the disturbance would be small because F120W and F120E retained marked catalytic activities.

Thermal Denaturation Properties of the Wild Type and Mutant RNase As—The denaturation profiles of the wild type and mutant enzymes appear to agree with a two state

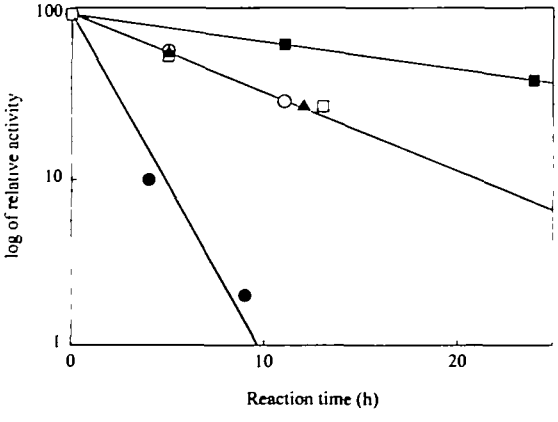

Fig. 3. **Different reactivities of the wild type and mutant RNase As with iodoacetate.** The wild type or a mutant RNase A was incubated with a 1,000 molar excess of iodoacetate at pH 5.5 and 25°C. The activity toward CpA was measured at various times (●, wild type; ○, F120L; ■, F120W; □, F120A; ▲, F120E).

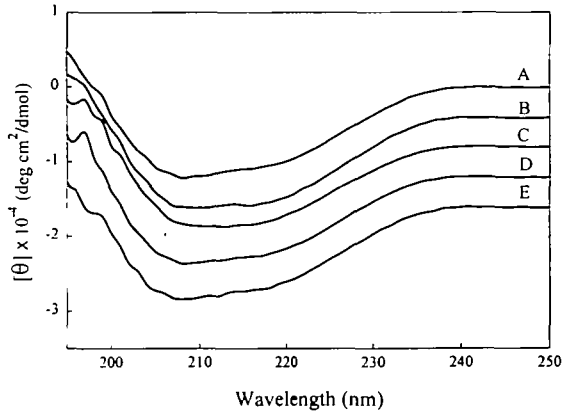

Fig. 4. **Far-UV CD spectra of the wild type and mutant RNase As.** The spectra of wild type or mutant RNase As were obtained by subtracting the following $[\theta]$ values from each ordinate: 4,000 for F120L, 8,000 for F120W, 12,000 for F120A, and 16,000 for F120E. Lines A, B, C, D, and E show the spectra of the wild type RNase A, F120L, F120W, F120A, and F120E, respectively.

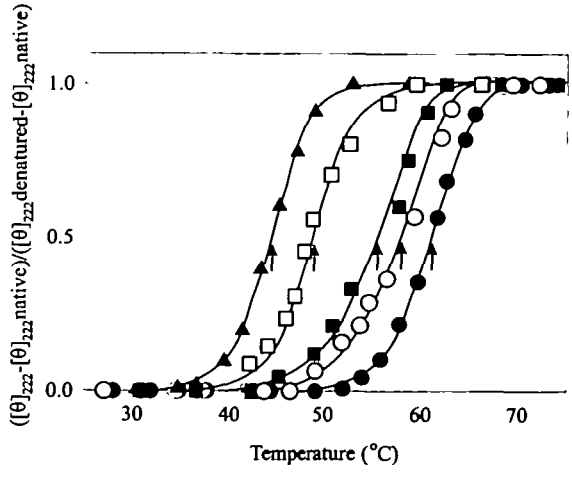

Fig. 5. **Thermal denaturation patterns of the wild type and mutant RNase As.** Thermal denaturation curves are shown for the wild type enzyme (●), F120L (○), F120W (■), F120A (□), and F120E (▲). The arrows indicate T_m values; 61°C for the wild type, 58°C for F120L, 56°C for F120W, 49°C for F120A, and 45°C for F120E.

transition model (Fig. 5). The thermal transition temperatures (T_m), the midpoint temperatures of thermal denaturation, are shown. The mutant enzymes, especially F120A and F120E, exhibited markedly lower T_m values than the wild type enzyme.

DISCUSSION

The time course of folding and the pattern of thermal denaturation (Figs. 1 and 5) indicate that hydrophilicity, such as the negative charge in F120E, reduces the folding rate and stability. As to the hydrophobicity of the residue introduced at position 120, the size of the hydrophobic side chain exhibits a linear correlation with the folding rate and thermal stability. For example, alanine which differs greatly in the van der Waals volume in comparison with phenylalanine, markedly reduced the folding rate and T_m value. In contrast, leucine or tryptophan, which have volumes similar to that of phenylalanine, gave similar folding rates and T_m values. The hydrophobicity and spatial volume of the phenyl ring therefore must be important for the folding rate and thermal stability of RNase A. This is supported by X-ray crystallography data which showed a hydrophobic core composed of Met13, Thr45, Val47, Val54, Ile81, Ile106, Val108, and Phe120 (*17*).

Although the folding rate and thermal stability changed markedly for the mutant enzymes, the similar properties found for the wild type and mutant enzymes, *i.e.* the CD spectra and pronounced catalytic activities of the mutant enzymes, indicate that their folded structures are identical to the structure of the wild type. This is relevant to the following discussion.

Role of Phe120 in Substrate Binding—RNase A recognizes ribonucleotide substrates *via* two types of binding sites, base and phosphate anion binding sites. The X-ray crystallographic study showed that a pyrimidine base binding site, one of the base-binding sites, is composed of Thr45, Phe120, and Ser123, and that the p_1-site, one of the phosphate anion binding sites, is composed of His12, Lys41, and His119.

As judged by the large changes in the K_m values of the mutant enzymes (Table II), a critical factor for substrate binding may be the space available for the pyrimidine base: A residue of adequate size is required at position 120 for proper substrate binding. An alanine residue would be too small to fill the space whereas a tryptophan residue, which has a large indole ring, would interfere with pyrimidine base binding. Because the hydrophilicity introduced at position 120 reduced the substrate binding capacity, as shown by the increased K_m of F120E, another important requirement for the pyrimidine base binding is a hydrophobic environment. The importance of the hydrophobic interaction between phenylalanine and the pyrimidine base has been reported by Eftink and Biltonen (*24*), who showed that protonation of the 3′ imino group of the cytidine base ($pK = 4.05$) markedly affects substrate binding.

The small changes in the K_I values suggest that the p_1-binding site is little affected by the mutation. The change in K_I is minor compared to that in pyrimidine base-binding ability.

Our findings indicate that the reduced substrate-binding ability of the mutant enzymes is most probably due to decreased ability to bind to the pyrimidine base.

Role of Phe120 in Construction of the Catalytic Center—The amino acid replacement of Phe120 affects both the K_m and k_{cat} values. The mutation effects on k_{cat}^{CpA} and $k_{cat}^{C>p}$ are different, as seen in the increased $k_{cat}^{CpA}/k_{cat}^{C>p}$ values for the mutant enzymes (Table II). There are three possible explanations for the role of Phe120 in catalysis: juxtapositioning of the two catalytic histidine residues; pK_a changes in the two catalytic histidinyl imidazole groups; and stabilization of the transition state intermediate during the C>p hydrolysis reaction.

1) Juxtapositioning of the two catalytic histidine residues: Alkylation of RNase A with iodoacetate at pH 5.5 yields 1-carboxymethyl His119 RNase A and 3-carboxymethyl His12 RNase A in the ratio of 8:1 (*2*). In this reaction, the protonated histidine residue interacts with the anionic portion of the iodoacetate, causing alkylation of the unprotonated histidine residue. The orientations of the two imidazole groups therefore are critical as to the rate and specific ratio of the two alkylation products. The extensively reduced alkylation rate for the mutant enzymes (Fig. 3) shows that Phe120 is an important residue juxtaposing the His12 and His119 residues.

The X-ray crystallographic data for RNase A show that the phenyl ring of Phe120 is close and almost parallel to the imidazole ring of His12, whereas the phenyl ring of Phe120 is situated far from the imidazole ring of His119. It is reasonable to assume that direct interaction between the phenyl ring of Phe120 and the imidazole ring of His12 in the wild type RNase A is disturbed by a mutation.

As a His12 proton is donated directly to the 2′-oxygen atom of the ribose in the hydrolysis reaction (*3, 25*), the imidazole ring of His12 must be fixed in a particular position. In contrast, the imidazole ring of His119 must be loosely fixed during hydrolysis, because nucleophilic attack by His119 occurs *via* a water molecule. The disturbance of the imidazole ring orientation of His12 explains why the mutant enzymes showed bigger decreases in the hydrolysis than the transphosphorylation reaction.

Specific interaction between Phe120 and the positive charge on either of the two histidine residues may create electrical properties and spatial orientations of the catalytic residues suitable for optical catalysis. Recent reports suggested there are specific interactions of phenylalanine or other aromatic residues with a positively charged amino acid residue or substrate. In the acetylcholine receptor, a cluster of aromatic residues comprises the binding site for acetylcholine, in which a positive charge exists on a nitrogen atom (*26, 27*). In barnase, Trp94, which is positioned near the catalytic histidine residue, His18, stabilizes a protonated form of that residue, the distance between the indole and imidazole rings being 4 Å and the angle between them being 43° (*28*). The X-ray crystallographic data for bovine pancreatic RNase A, however, show that the distance between the phenyl ring of Phe120 and the imidazole ring of His12 is about 5 Å, and that they are parallel. This parallel orientation of the two rings is inconvenient for optical interaction between the π electron and the positive charge. Phe120 therefore makes no, or little, contribution to this kind of interaction with His12. Instead, the parallel angle between the imidazole ring of His12 and the phenyl ring of Phe120 suggests that the stacking interaction between the two rings may be the force that keeps His12 in the right position.

2) pK_a changes in the two catalytic histidynyl imidazole groups: No, or only slight, changes in the pK_a values of the histidynyl imidazole group took place when phenylalanine was replaced with leucine or alanine, as inferred from the pK_e values (Fig. 2). This suggests that Phe120 makes a negligible contribution to the pK_a values of the catalytic histidine residues. The increased pK_{e1} values of F120E and F120W suggest that both a negative charge on the glutamic acid residue and the π electron of the tryptophan residue interact with the protonated form of His12.

Increased $k_{cat}^{CpA}/k_{cat}^{C>p}$ was common among the mutant enzymes (Table II). We expected that this increase would be due to the decreased pK_{e1} value due to His12; but the results indicate that the pK_{e1} values of the mutants are maintained or increased. Therefore, the change in the pK_a value of His12 is not the main cause of the increase in $k_{cat}^{CpA}/k_{cat}^{C>p}$.

3) Stabilization of the transition state during the C>p hydrolysis reaction: The replacement of Phe120 more markedly affected the k_{cat} values for the hydrolysis reaction than those for the transphosphorylation reaction (Table II). To determine why, we considered the conformational change in the pyrimidine nucleotide during hydrolysis.

As the glycosidic bond of C>p is fixed in the *syn* conformation in solution (*29*), and that of 3′ CMP is in the *anti* conformation while it is bound to RNase A (see Fig. 6), the pyrimidine ring rotates from the *syn* to *anti* conformation during hydrolysis. This rotation, however, is unfavorable energy wise, because C>p takes on a rigid conformation due to intramolecular interactions (see Fig. 6). The conformational change from *syn* to *anti* would be induced when the pyrimidine base interacts tightly with the amino acid residue at position 120. The stacking interaction between the pyrimidine base and phenyl ring, which does not take place in the mutant enzymes, would stabilize the transition state intermediate in the hydrolysis reaction leading to an increased k_{cat} value for C>p.

This is supported by the fact that the wild type RNase A catalyzes transphosphorylation about 670 times faster than it does hydrolysis (Table 2). This is because His12 (pK_a = 5.8) acts as a base and His119 (pK_a = 6.2) as an acid in the transphosphorylation reaction. Our findings, however, show that the catalytic preferences are not necessarily correlated with these changes in the pK_a values (see above).

The catalytic preference can be explained by the difference in the glycosidic conformation of the substrate: CpA bound by RNase A has an *anti* glycosidic bond, as suggested by the structure of the substrate analog, d(CpA) and RNase A complex (*17*), whereas C>p has a *syn* glycosidic bond. Because the transition state intermediate requires the *anti* conformation during both reactions, the energy for transition may be lower in transphosphorylation than in hydrolysis. Replacement of Phe120 therefore results in a small change in the k_{cat} value for the transphosphorylation reaction.

In conclusion, the volume and hydrophobicity of Phe120 are important for the correct orientation of His12, and for stabilization of the transition state intermediate during hydrolysis. Nevertheless, the replacement of Phe120 has only a slight effect on the catalysis speed. This suggests that substrate binding to RNase A may function in the juxtapositioning of the imidazole ring of His12 for effective catalysis.

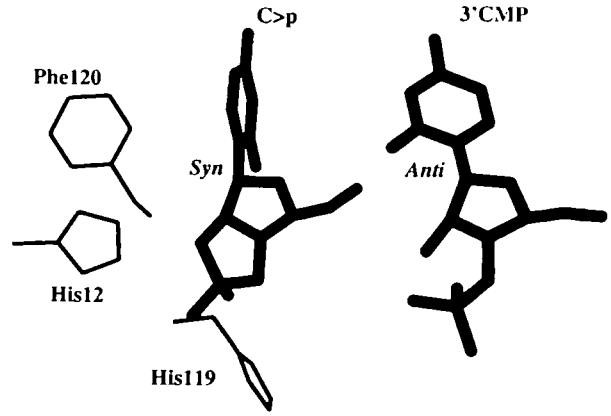

Fig. 6. **Structures of C>p and 3′CMP.** The structure of 3′CMP is taken from Zegers *et al.* (*17*), and that of C>p was designed with a molecular editor program, with reference to Coulter (*29*). In general, the glycosidic bond between the pyrimidine base and ribose exists in a mixture of the *syn* and *anti* conformations. In the *syn* conformation, the 2-carbonyl oxygen of the pyrimidine ring lies just above the ribose ring, and in the *anti* conformation, the 2-carbonyl oxygen protrudes away from the ribose ring. The glycosidic bond of C>p has the *syn* conformation, in which an intramolecular interaction exists between the 2-carbonyl oxygen of the pyrimidine base and the covalent bond between the 2′ and 3′ oxygens of the ribose ring *via* the phosphorus atom. The glycosidic bond of 3′CMP has the *anti* conformation when it is bound to RNase A.

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