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Structural Determinants of Enzymatic Processivity†

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ABSTRACT: A processive enzyme binds a polymeric substrate and catalyzes a series of similar chemical reactions along that polymer before releasing the fully modified polymer to solvent. Bovine pancreatic ribonuclease A (RNase A) is a nonprocessive endoribonuclease that binds the bases of adjacent RNA residues in three enzymic subsites: B1, B2, and B3. The B1 subsite binds only to residues having a pyrimidine base, while the B2 subsite prefers adenine and the B3 subsite prefers a purine base. RNase A mutants were created in which all natural amino acids were substituted for Thr45 or Phe120, two residues of the B1 subsite. These pools of mutant enzymes were screened for mutants that catalyze the cleavage of RNA after purine residues. The Ala45 and Gly45 enzymes cleave poly(A), poly(C), and poly(U) efficiently and with 10^3–10^5-fold increases in purine/pyrimidine specificity. Thus, substrate binding can be uncoupled from substrate turnover in catalysis by RNase A. In addition, both mutant enzymes cleave poly(A) processively. Our results provide a new paradigm: a processive enzyme has subsites, each specific for a repeating motif within a polymeric substrate. Further, we propose that processive enzymes bind more tightly to motifs that do repeat than to those that do not.

During enzymatic catalysis, noncovalent and covalent bonds are formed and broken. Upon binding, an enzyme and its substrate exchange noncovalent interactions with solvent and solute molecules for interactions with each other. During turnover, a medley of devices is used to effect changes in the covalent bonds of the bound substrate (Jencks, 1987). Altering the substrate specificity of an enzyme requires adjusting interactions that mediate substrate binding but not those critical to substrate turnover. Impressive results have been obtained in the redesign of several proteases (Craik et al., 1985; Estell et al., 1986; Wells et al., 1987; Bone et al., 1989; Rheinnecker et al., 1993) and some dehydrogenases (Wilks et al., 1988; Scrutton et al., 1990). For many enzymes, however, binding and turnover are coupled irrevocably, as the same amino acid residues mediate both (Knowles, 1987). Thus, mutant enzymes of altered specificity are often relatively inefficient catalysts.

In addition to changing substrate specificity, mutations to active-site residues can, in principle, also affect the *mechanism* by which an enzyme binds to its substrate. Examples of different mechanisms for binding substrates are found among enzymes that modify biopolymers. *Distributive* enzymes bind a polymeric substrate, catalyze a chemical reaction, and release to solvent a polymeric product. In contrast, *processive* enzymes bind a polymeric substrate and catalyze a series of identical chemical reactions along that polymer before releasing it to solvent (Kornberg & Baker, 1992).

Bovine pancreatic ribonuclease A (RNase A;[1] EC 3.1.27.5) is a small protein (14 kDa) that has been an exemplar for studies in all aspects of protein chemistry and enzymology (Richards & Wyckoff, 1971; Karpeisky & Yakovlev, 1981; Blackburn & Moore, 1982; Wlodawer, 1985; Beintema, 1987; Eftink & Biltonen, 1987). The enzyme has also been of particular use in biotechnology (Zuckermann & Schultz, 1988; Kim & Raines, 1993b). RNase A is a distributive endori-

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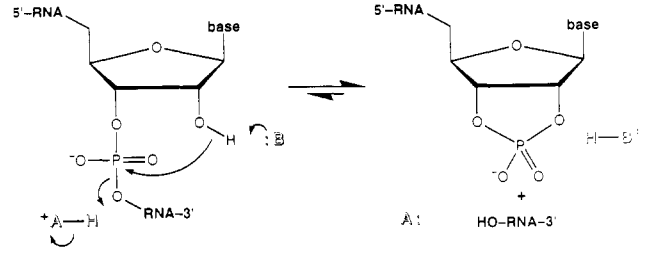

FIGURE 1: Mechanism of the cleavage reaction catalyzed by RNase A. B is His12; A is His119 (Thompson & Raines, 1994).

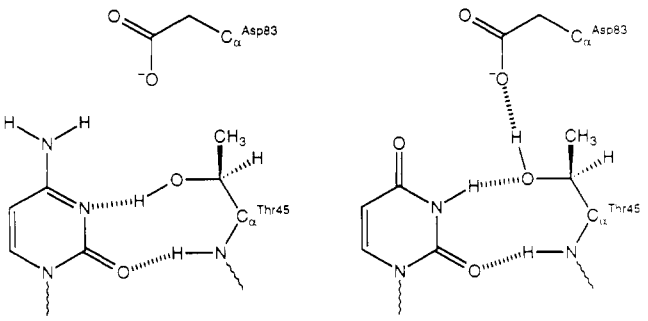

FIGURE 2: Hydrogen bonds formed between the pyrimidine bases C (left) and U (right) and residues of the B1 binding pocket of RNase A (Wlodawer & Sjölin, 1983).

bonuclease that binds the bases of adjacent RNA residues in three enzymic subsites: B1, B2, and B3 (Parés et al., 1991). Catalysis by RNase A results in the cleavage of the P–$O_{5'}$ bond specifically on the 3′-side of pyrimidine nucleotides that are bound in the B1 subsite (Figure 1). Indeed, the B1 subsite binds only residues having a pyrimidine base (McPherson et al., 1986; Aguilar et al., 1992; Fontecilla-Camps et al., 1993). In contrast, the B2 and B3 subsites bind all residues, but B2 has a preference for those having an adenine base (Katoh et al., 1986) and B3 has a preference for those having a purine base (Rushizky et al., 1961; Irie et al., 1984). After cleavage of the P–$O_{5'}$ bond, the 2′,3′-cyclic phosphodiester product of transphosphorylation (Figure 1) is released from the enzyme to be hydrolyzed in a slow, separate step (Thompson et al., 1994).

Structural (Wlodawer & Sjölin, 1983; Santoro et al., 1993) and phylogenetic (Beintema, 1987) data suggest that the specificity of the B1 subsite is mediated by the side chains of Thr45 and Phe120.[2] The hydroxyethyl side chain of Thr45 forms hydrogen bonds with a pyrimidine base (U or C; Figure 2) and excludes sterically the purine bases (A and G). The benzylic side chain of Phe120 makes van der Waals contact with a pyrimidine base and with the side chain of Thr45.

We are interested in the molecular determinants of substrate specificity and in how substrate specificity effects processivity in enzymatic catalysis. To search for RNase A mutants that cleave RNA after purine residues, we screened pools of enzymes in which all natural amino acids had been substituted for either Thr45 or Phe120. We found that mutating residue 45 of RNase A to alanine or glycine expands the specificity of the B1 subsite to include residues with an adenine base. In addition, we discovered that although the cleavage of poly(U) and poly(C) by T45G, T45A, or wild-type RNase A is distributive, the cleavage of poly(A) by either mutant enzyme is processive.

EXPERIMENTAL PROCEDURES

Materials. Poly(A), poly(C), poly(G), poly(U), UpA, ApA, A>p, and C>p were from Sigma Chemical Co. (St. Louis, MO). Poly(C) and poly(U) were from Midland Certified Reagents (Midland, TX). UpA was synthesized by J. E. Thompson using the methods of Ogilvie et al. (1978) and Beaucage and Caruthers (1981). Polymeric substrates were precipitated from aqueous ethanol (70% v/v) before use. [γ-^{32}P]ATP was from Amersham (Arlington Heights, IL). T4 RNA kinase was from United States Biochemicals (Cleveland, OH). All enzymes for the manipulation of DNA were from Promega (Madison, WI). Expression vector pET22B(+) and *Escherichia coli* strain BL21(DE3) were from Novagen (Madison, WI).

Reagents for DNA synthesis were from Applied Biosystems (Foster City, CA), except for acetonitrile, which was from Baxter Healthcare (McGaw Park, IL). Agarose was from Life Technologies (Gaithersburg, MD). Acrylamide and *N*,*N*′-methylenebis(acrylamide) were from Bio-Rad (Richmond, CA). All other chemicals and reagents were of commerical reagent grade or better and were used without further purification.

General Methods. Ultraviolet absorbance measurements were made on a Cary Model 3 spectrophotometer equipped with a Cary temperature controller. Substrate concentrations were determined by ultraviolet absorption using ϵ_{260} = 24 600 M^{-1} cm^{-1} at pH 7.0 for UpA (Warshaw & Tinoco, 1966), ϵ_{257} = 10 000 M^{-1} cm^{-1} at pH 7.5 for poly(A), ϵ_{268} = 6200 M^{-1} cm^{-1} at pH 7.8 for poly(C), and ϵ_{261} = 9430 M^{-1} cm^{-1} at pH 7.5 for poly(U) (Yakovlev et al., 1992) and are given in terms of the molarity of P–$O_{5'}$ bonds. DNA oligonucleotides were synthesized on an Applied Biosynthesis Model 392 DNA/RNA synthesizer by using the β-cyanoethyl phosphoramidite method (Sinha et al., 1984). DNA sequences were determined with the Sequenase Version 2.0 kit from United States Biochemicals. Manipulations of RNA and DNA were performed as described (Ausubel et al., 1989).

Molecular models of RNase A mutants were made from the coordinates of the crystalline complex of RNase A with uridine 2′,3′-cyclic vanadate (Wlodawer & Sjölin, 1983) with the program MIDAS PLUS (Ferrin et al., 1988). Mutant proteins were made by replacing the side chain of residues 45 and 120. Purine nucleotidyl vanadates were made by replacing the uracil base. No calculations were performed to minimize energy.

Mutagenesis. Plasmid pBXR directs the production of RNase A in *E. coli* (delCardayré and Raines, unpublished results). This plasmid was constructed by inserting the cDNA that codes for RNase A (Raines & Rutter, 1989) between the *Msc*I and *Sal*I sites in expression vector pET22B(+). Oligonucleotide-mediated site-directed mutagenesis (Kunkel et al., 1987) of plasmid pBXR was used to introduce a unique and translationally silent *Nhe*I site into the cDNA for RNase A on the 3′-side of the codon for Thr45, resulting in plasmid pBXR1. The codon for Thr45 was then randomized by cassette-mediated saturation mutagenesis (Reidhaar-Olson et al., 1991) of the *Cla*I/*Nhe*I fragment in pBXR1. The codon for Phe120 was randomized by cassette mutagenesis of the *Bsi*WI/*Sal*I in pBXR1. The oligonucleotides used were

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; poly(A), poly(adenylic acid); poly(C), poly(cytidylic acid); poly(G), poly(guanylic acid); poly(U), poly(uridylic acid); ApA, adenylyl(3′→5′)-adenosine; UpA, uridylyl(3′→5′)adenosine; A>p, adenosine 2′,3′-cyclic phosphate; C>p, cytidine 2′,3′-cyclic phosphate; U>p, uridine 2′,3′-cyclic phosphate; NMR, nuclear magnetic resonance.

[2] Thr45 is conserved in pancreatic ribonucleases sequenced from 41 species. Phe120 is conserved in 36 species (5 Tyr). No homologs of RNase A are known to catalyze the efficient cleavage of RNA after purine residues.

CGATGCAAGCCAGTGAACNN(G/C)TTTGTGCAC-GAGTCG and CTAGCGACTCGTGCACAAA(G/C)N-NGTTCACTGGCTTGCAT for Thr45All and CTACGT-GCCAGTCCACNN(G/C)GATGCTTCAGTGTAG and TCGACTACACTGAAGCATC(G/C)NNGTGGACTG-GCAC for Phe120All. Individual clones from the Thr45All and Phe120All cDNA libraries were sequenced to discern the randomness of codons 45 and 120.

Screen for Mutants of Altered Specificity (delCardayré et al., 1994). Culture medium from *E. coli* BL21(DE3) cells expressing the cDNA that codes for Thr45All or Phe120All was screened by zymogram electrophoresis (Blank et al., 1982; Ribó et al., 1991; Kim & Raines, 1993a) for production of an enzyme able to cleave poly(A) or poly(G). Culture medium (15 μL) was separated by nonreducing SDS–PAGE in a running gel copolymerized with poly(A), poly(C), or poly(G) (0.5 mg/mL). After electrophoresis, proteins in the gel were renatured by washing the gel (for 2 × 10 min) with 10 mM Tris·HCl buffer, pH 7.5, containing 2-propanol (20% v/v) to extract the SDS, and then (for 2 × 20 min) with 10 mM Tris·HCl buffer, pH 7.5. The gel was stained (for 5 min) with 10 mM Tris·HCl buffer, pH 7.5, containing toluidine blue (0.2% w/v), which binds tightly to high molecular weight nucleic acid, and then destained with water. Regions in the gel containing ribonuclease activity appear as clear bands in a blue background.

The mutant enzymes from the Thr45All library that were responsible for the observed poly(A) cleaving activity were identified by using a zymogram spot assay. Zymogram spot assays involved placing samples (1 μL) of culture medium from individual clones on an agarose gel (1% w/v) containing poly(A) (0.3 mg/mL) and 10 mM Tris·HCl buffer, pH 7.5. The gel was then incubated at 37 °C for 4 h before being stained as above. Plasmid DNA from any clones showing poly(A) cleaving activity was isolated and sequenced. Active enzymes were purified to homogeneity from the insoluble fraction of cell lysates by methods similar to those of McGeehan and Benner (1989).

Steady-State Kinetics. The cleavage of poly(U), poly(C), or poly(A) was monitored by the decrease in ultraviolet hypochromicity. The $\Delta\epsilon$ values for these reactions, calculated from the difference in molar absorptivity of the polymeric substrate and the mononucleotide cyclic phosphate product, were 1360 M^{-1} cm^{-1} for poly(U) at 278 nm, 2380 M^{-1} cm^{-1} for poly(C) at 250 nm, and 6400 M^{-1} cm^{-1} for poly(A) at 260 nm. The cleavage of UpA was monitored with an adenosine deaminase coupled assay (Ipata & Felicioli, 1968). The $\Delta\epsilon$ for this reaction was –6000 M^{-1} cm^{-1} at 265 nm. RNase A concentrations were determined by assuming that $A_{278}^{0.1\%} = 0.71$ (Richards & Wyckoff, 1971). Assays were performed at 25 °C in 0.1 M MES buffer, pH 6.0, containing NaCl (0.1 M), substrate (10 μM to 0.75 mM), and enzyme (1.0 nM to 1.0 μM). The values of k_{cat}, K_m, and k_{cat}/K_m were determined from initial velocity data with the program HYPERO (Cleland, 1979).

^{31}P NMR Assay for Processivity. NMR assays were performed at 25 °C in 0.1 M MES buffer, pH 6.0, containing NaCl (0.1 M), RNA (5.0 mM), and enzyme (1.0–50 μM). Each reaction was monitored for 50 min. Free induction decays were obtained on a Bruker AM400 spectrophotometer in 10-mm NMR tubes having D_2O inserts using the following parameters: 4854-Hz spectral width, 90° pulse width, 1.69-s acquisition time, 3.2-s relaxation time, and 64 scans. The free induction decays were subjected to Fourier transformation with a line broadening of 5 Hz. The resulting spectra were phased with the program FELIX (Hare Research, Bothell, WA). Chemical shift values were recorded relative to 0.1 M H_3PO_4. Resonances were assigned on the basis of the spectra of the purified reagents: poly(A), poly(A)A>p, ApA, A>p, poly(C), and C>p.

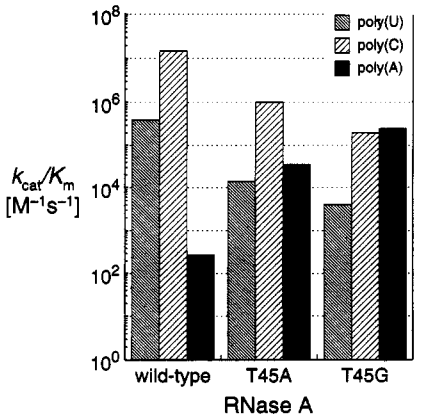

FIGURE 3: Specificity constants of wild-type and mutant RNase A for the cleavage of homopolymeric substrates.

Distraction Assay for Processivity. An order-of-addition experiment was used to determine whether preincubation with unlabeled RNA could distract T45A, T45G, or wild-type RNase A from degrading 5′-^{32}P-labeled RNA. In all distraction assays, the unlabeled substrate was identical to the labeled substrate except for the presence of the [5′-^{32}P]-PO_3^{2-} group. [For example, we determined whether poly(A) could distract the enzyme from cleaving [5′-^{32}P]poly(A).] Unlabeled substrate (15 nM) was exposed to enzyme (1.5 nM) for time t' to allow any processive complex to form. 5′-^{32}P-labeled substrate (15 nM) was then added, and the mixture was incubated for an additional time t before being quenched. Assays were performed in 0.1 M MES buffer, pH 6.0, containing NaCl (0.1 M), and were quenched by 10-fold dilution into 95% v/v formamide containing xylene cyanol (0.05% w/v). The reaction products were separated by electrophoresis in a gel of polymerized acrylamide [7.5% w/v in 90 mM Tris·H_3BO_3 buffer, pH 7.6, containing EDTA (2 mM) and urea (8 M)] and visualized by autoradiography.

RESULTS

Screen for Mutants of Altered Specificity. The sequence of individual clones from the Thr45All and Phe120All cDNA libraries was determined. Codons 45 and 120 were considered to be random because G, A, T, and C were found in each of the first two positions and G and C were found in the third position of these codons. Screens (by zymogram electrophoresis) of culture medium from *E. coli* cells expressing the mutant libraries showed that a polypurine-cleaving activity was present only in the Thr45All pool and that this activity was for cleavage of poly(A) but not for cleavage of poly(G). Screens (by zymogram spot assay) of 100 individual clones from the Thr45All pool identified 8 clones that produced an enzyme capable of cleaving poly(A) efficiently. Sequencing of plasmid DNA from these clones revealed that each active mutant had either an alanine or a glycine residue at position 45.

Steady-State Kinetics. Steady-state kinetic parameters for the cleavage of the homopolymers poly(A), poly(C), and poly(U) as well as for the diribonucleotide UpA were determined for wild-type, T45A, and T45G RNase A. As the side chain of residue 45 became smaller (Thr → Ala → Gly), the value of the specificity constant, k_{cat}/K_m (Fersht, 1985), for poly(A) cleavage became larger (Figure 3). This increase was a

Table 1: Steady-State Kinetic Parameters for Cleavage of Ribonucleotides by Wild-Type, T45A, and T45G RNase A

	k_{cat} (s^{-1})				K_m (mM)			
RNase A	UpA	poly(U)	poly(C)	poly(A)	UpA	poly(U)	poly(C)	poly(A)
wild type	1400 ± 150	24 ± 15	510 ± 10	0.023 ± 0.001	0.62 ± 0.09	0.06 ± 0.01	0.034 ± 0.002	0.080 ± 0.009
T45A	24 ± 13	1.7 ± 0.2	500 ± 60	1.4 ± 0.1	4 ± 2	0.12 ± 0.04	0.48 ± 0.08	0.041 ± 0.005
T45G	20 ± 10	0.86 ± 0.08	1000 ± 300	5.8 ± 0.2	6 ± 4	0.19 ± 0.04	4 ± 2	0.023 ± 0.004

	k_{cat}/K_m (10^6 M^{-1} s^{-1})			
RNase A	UpA	poly(U)	poly(C)	poly(A)
wild type	2.3 ± 0.4	0.4 ± 0.3	15 ± 1	0.00028 ± 0.00004
T45A	0.006 ± 0.005	0.014 ± 0.005	1.0 ± 0.2	0.035 ± 0.005
T45G	0.0030 ± 0.0025	0.004 ± 0.001	0.2 ± 0.1	0.25 ± 0.05

RNase A	$(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(U)}$	$((k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(U)})_{rel}$	$(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(C)}$	$((k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(C)})_{rel}$
wild type	$(7.0 \pm 4.7) \times 10^{-4}$	1	$(1.9 \pm 0.3) \times 10^{-5}$	1
T45A	2.5 ± 1.0	$(4 \pm 3) \times 10^3$	0.035 ± 0.009	$(1.8 \pm 0.5) \times 10^3$
T45G	62 ± 19	$(9 \pm 7) \times 10^4$	1.2 ± 0.6	$(6 \pm 3) \times 10^4$

result of both an increase in k_{cat} and a decrease in K_m (Table 1). The T45G mutant actually cleaved poly(A) more efficiently than it cleaved poly(U) or poly(C), with $(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(U)}$ = 62 [(9×10^4)-fold change in substrate specificity] and $(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(C)}$ = 1.2 [(6×10^4)-fold change]. This reversal of specificity likely results from a broadening of the specificity of the B1 subsite and the inherent preference of the B2 and B3 subsites for purine residues. The T45A mutant exhibited a 20–30-fold smaller change in substrate specificity than did the T45G mutant, with $(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(U)} = 2.5$ [(4×10^3)-fold change] and $(k_{cat}/K_m)_{poly(A)}/(k_{cat}/K_m)_{poly(C)} = 0.035$ [(2×10^3)-fold change]. Since the relative preference of the wild-type enzyme for poly(C) and poly(U) is maintained in the T45A and T45G mutants (Figure 3), this preference must not be mediated by Thr45 in RNase A. [The role of the active-site Thr residue in mediating this preference in homologs of RNase A may be more pronounced (Miranda, 1990; Curran et al., 1993).] Although both mutant enzymes cleave polymeric substrates efficiently, both also have a markedly diminished ability to bind and turnover UpA (Table 1). This decrease is probably due to the loss of a large fraction of the total binding energy of the enzyme for this small substrate.

Molecular Modeling. Models of T45A and T45G RNase A showed that truncating the side chain of Thr45 creates a cavity that can accommodate adenine. Guanine cannot fit in this cavity due to unfavorable steric interactions of N2 with the main chain of residue 45. The carboxyl group of Asp83, which is also in the B1 subsite (Figure 2), may be responsible for the preference of the wild-type and mutant enzymes for poly(C) versus poly(U) (Figure 3).

Processive Catalysis by RNase A. RNase A is known to have at least three subsites that bind to bases in polymeric RNA (McPherson et al., 1986; Parés et al., 1991; Fontecilla-Camps et al., 1993). Thr45 contributes to the B1 subsite, which is highly specific for pyrimidine bases as discussed above. The B2 subsite binds to the base 3′ to that in B1 and, while accepting all bases, has a preference for adenine. The B3 subsite binds to the base 3′ to that in B2 and, while also accepting all bases, has a preference for purines. Engineering RNase A to accept adenine bases in the B1 subsite has allowed poly(A) to satisfy the base specificity of all three subsites. Once cleavage between the residues occupying the B1 and B2 subsites occurs and one strand is released, the T45A and T45G enzymes could refill the B1, B2, and B3 subsites by moving down one (in the 5′ → 3′ direction; Figure 4) or two (in the 3′ → 5′ direction) residues of the poly(A) strand. We therefore

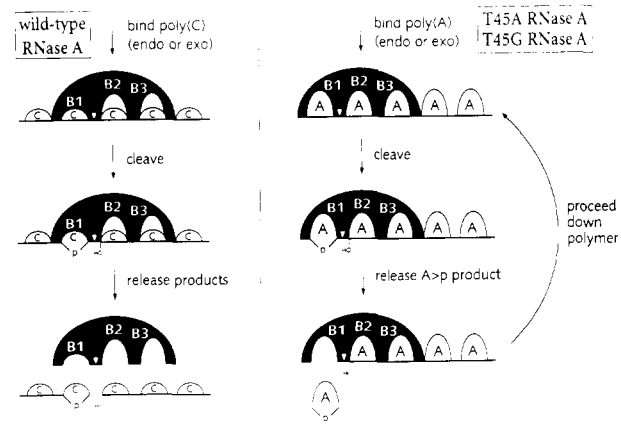

FIGURE 4: Models for distributive and processive cleavage of RNA by wild-type and mutant RNase A. Wild-type RNase A cleaves poly(C) distributively, releasing both RNA cleavage products. In contrast, the T45A and T45G enzymes cleave poly(A) processively, releasing only the A>p product and remaining bound to the polymeric product after each cleavage reaction. The model for processive cleavage implies that the mutant enzymes proceed in the 5′ → 3′ direction, which is most consistent with the alignment of the subsites and with data not shown.

reasoned that the mutant enzymes may degrade a strand of poly(A) in a processive rather than a distributive manner.

^{31}P NMR Assay for Processivity. ^{31}P NMR spectroscopy can be used to probe the chemical state of the phosphoryl group during RNA cleavage (that is, acyclic diester → cyclic diester) (Cazzone & Jardetsky, 1977; Thompson et al., 1994). Further, the relative molecular weight (strand length) of a phosphodiester can be inferred from its ^{31}P NMR chemical shift. If an acyclic diester [such as poly(A)] were being cleaved processively, then the spectra would show no accumulation of high molecular weight polymers containing a cyclic diester [poly(A)A>p] and no decrease in the molecular weight of the acyclic diester. Instead, the acyclic diester would be converted completely to a monomeric cyclic diester (A>p). If the substrate were not being cleaved processively, then the spectra would shown an accumulation of polymeric cyclic diesters and a decrease in the molecular weight of the acyclic diester.

Spectra for the degradation of poly(A) and poly(C) by T45G and wild-type RNase A are shown in Figure 5. During the degradation of poly(C) by the mutant and wild-type enzymes [or of poly(A) by the wild-type enzyme (Cazzone & Jardetsky, 1977)], the ^{31}P resonance of the acyclic diester shifted from −1.30 to −0.90 ppm. This downfield shift arises from the deshielding of the phosphorus atom in low molecular weight

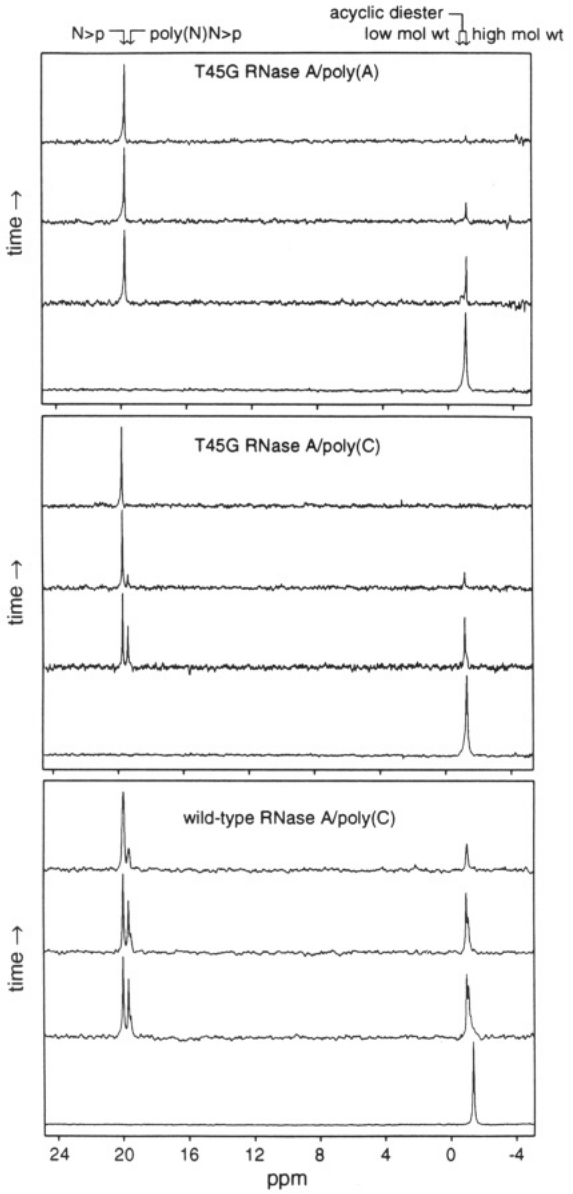

FIGURE 5: Spectra showing ^{31}P NMR assay for processivity. Panels show spectra recorded during cleavage of poly(C) by wild-type RNase A (bottom), poly(C) by T45G RNase A (middle), and poly(A) by T45G RNase A (top).

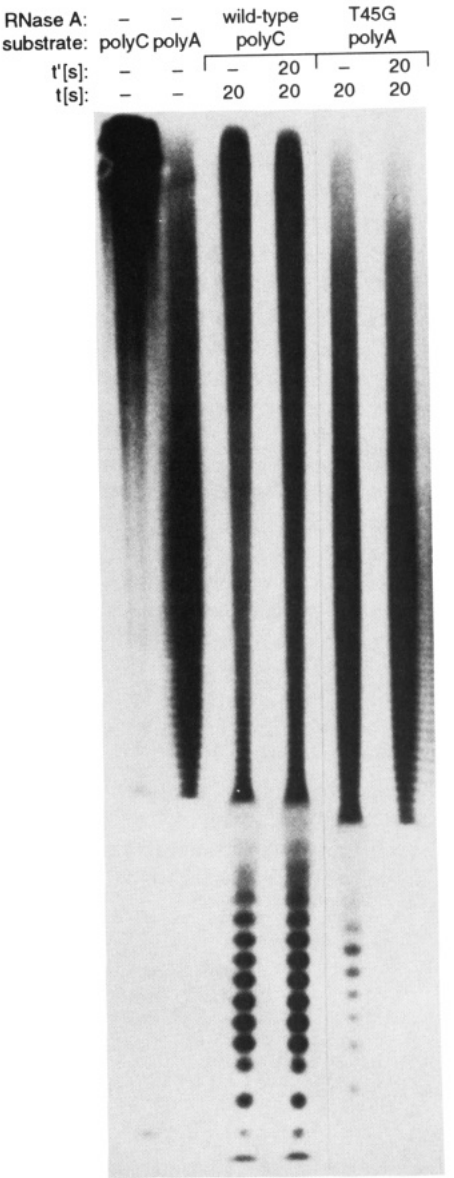

FIGURE 6: Gel showing distraction assay for processivity. Unlabeled substrate was exposed to enzyme for time t' to allow any processive complex to form. 5′-^{32}P-labeled substrate was then added, and the mixture was incubated for an additional time t before being quenched.

polymers. Concurrent with the shift in the resonance of acyclic diesters was the appearance of two resonances from cyclic diesters, one at 19.7 ppm [from poly(C)C>p] and another at 20.1 ppm (from C>p). The accumulation of poly(C)C>p and the decrease in molecular weight of the acyclic diester are consistent with a distributive degradation of poly(C). In contrast, during the degradation of poly(A) by the T45G enzyme, the resonance from the acyclic diester remained at –1.03 ppm, indicating that most strands of poly(A) maintained a constant molecular weight. This result is inconsistent with either endonucleolytic or distributive exonucleolytic degradation, which would have resulted in a downfield shift of the acyclic diester resonance. In addition, a single cyclic diester resonance appeared at 19.9 ppm (from A>p). The absence of poly(A)A>p along with the absence of low molecular weight acyclic diesters indicates that T45G RNase A degrades poly(A) strands one at a time to A>p and thus acts processively. Spectra (not shown) for cleavage by T45A RNase A were similar to those for cleavage by T45G RNase A.

Distraction Assay for Processivity. A ribonuclease that degrades RNA processively binds to an RNA strand and remains associated with that strand until its degradation is complete. Other RNA strands added after the initial association should be protected from degradation for the lifetime of the initial enzyme–polymer complex.

Results from the distraction assay (Figure 6) show that T45G RNase A was distracted by preincubation with unlabeled poly(A), as evidenced by the absence of small molecular weight species. Yet, neither T45G (not shown) nor wild-type RNase A was distracted by preincubation with unlabeled poly(C), as evidenced by the accumulation of small molecular weight species. These data, along with the data from ^{31}P NMR spectroscopy, demonstrate that T45G RNase A catalyzes the distributive degradation of poly(C) and the processive degradation of poly(A). Gels (not shown) for distraction of the T45A enzyme were similar to those for distraction of the T45G enzyme.

DISCUSSION

The side chain of the residue (Thr45; Figure 2) that is largely responsible for the substrate specificity of RNase A

is relatively remote from the side chains of the residues (His12 and His119; Figure 1) that expedite cleavage of the P–$O_{5'}$ bond of RNA. This separation suggests that substrate binding can be uncoupled from substrate turnover in RNase A. Our results indicate that such uncoupling is indeed possible, as the T45A and T45G enzymes display a 10^3–10^5-fold change in purine/pyrimidine specificity with little compromise to catalytic efficacy (Figure 3). These changes result largely from a 10^2–10^3-fold increase in the specificity constant for cleavage of poly(A) (Table 1).[3]

In contrast, no alteration of Phe120 produced an enzyme that catalyzed the efficient cleavage of RNA after purine residues. This result is consistent with two structural features of Phe120 that are apparent in the RNase A–uridine 2′,3′-cyclic vanadate complex (Wlodawer & Sjölin, 1983). First, the π system of Phe120 appears to interact with that of a pyrimidine base bound in the B1 subsite. The structural difference between a pyrimidine base and a purine base is largely two dimensional, in the plane of the π system. Hence, the side chain of Phe120 is likely to enhance substrate binding but not to mediate purine/pyrimidine specificity. Second, the main-chain nitrogen atom of Phe120 forms a hydrogen bond with a nonbridging oxygen atom of the reacting phosphoryl group. Thus, even if the side chain of Phe120 did mediate substrate specificity, changing this residue may hamper catalysis.

Many enzymes that catalyze the synthesis and degradation of nucleic acids do so processively (Kornberg & Baker, 1992). Further, several viral enzymes that catalyze these fundamental processes are noted for their exceptional processivity, which may provide these viruses with a competitive advantage. Unfortunately, little structural information is available on processive enzymes, leaving the molecular determinants for enzymatic processivity unclear. Inducing processivity in RNase A has illuminated these determinants.

For a substrate to be acted on processively, it must contain a repeating structural motif. Both poly(C) and poly(A) have repeating motifs, such as a ribosyl group, phosphoryl group, and base. Yet, neither of these polymers is cleaved processively by wild-type RNase A (Figures 5 and 6). The distributive behavior of RNase A likely arises from the opposing specificities of the B1 subsite [which does not bind A (McPherson et al., 1986)] and the B2 and B3 subsites [which bind C only weakly (Katoh et al., 1986)]. Our results show that inducing RNase A to degrade poly(A) processively requires simply changing the specificity of the B1 subsite to match that of the B2 and B3 subsites. This change results in mutant enzymes that cleave (at the B1 position) a polymer that can remain bound to the enzyme (at the B2 and B3 positions) after catalysis has occurred (Figure 4).

Apparently, processive enzymes must bind tightly only to those structural motifs of a polymer that do indeed repeat and must bind those motifs in more than one enzymic subsite. Conversely, enzymes that rely on the tight binding of nonrepeating motifs are likely to act in a distributive manner. Such binding interactions would prohibit processive catalysis. By considering these principles, it is possible to predict which structural motifs within a substrate must be bound tightly by a processive enzyme. For example, an enzyme that catalyzes the processive cleavage of a heteropolymer of RNA is likely to interact more strongly with the ribosyl or phosphoryl groups than with the bases. RNase II, a cytosolic enzyme from *E. coli*, may act in this way (Nossal & Singer, 1968).

[3] These U,C,A-specific mutants of RNase A may be of use in a ribonuclease protection assay (RPA), which is a sensitive technique for the detection, quantitation, and characterization of RNA. This assay is most often performed with a cocktail of RNase A and RNase T1, two stable enzymes that together cleave RNA after U, C, and G (but not A) residues. A cocktail containing the T45G or T45A enzyme and RNase T1 could cleave RNA after all four residues and thereby lead to an improved RPA.

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REFERENCES

Aguilar, C. F., Thomas, P. J., Mills, A., Moss, D. S., & Palmer, R. A. (1992) *J. Mol. Biol. 224*, 265–267.
Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1989) *Current Protocols in Molecular Biology*, Wiley, New York.
Beaucage, S. L., & Caruthers, M. H. (1981) *Tetrahedron Lett. 22*, 1859–1862.
Beintema, J. J. (1987) *Life Chem. Rep. 4*, 333–389.
Blackburn, P., & Moore, S. (1982) *Enzymes 15*, 317–433.
Blank, A., Sugiyama, R. H., & Dekker, C. A. (1982) *Anal. Biochem. 120*, 267–275.
Bone, R., Silen, J. L., & Agard, D. A. (1989) *Nature 339*, 191–195.
Cazzone, P. J., & Jardetsky, O. J. (1977) *FEBS Lett. 73*, 77–79.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.
Craik, C. S., Largman, C., Fletcher, T., Roczniak, S., Barr, P. J., Fletterick, R., & Rutter, W. J. (1985) *Science 228*, 291–297.
Curran, T. P., Shapiro, R., & Riordan, J. F. (1993) *Biochemistry 32*, 2307–2313.
delCardayré, S. B., Thompson, J. E., & Raines, R. T. (1994) in *Techniques in Protein Chemistry V* (Crabb, J. W., Ed.) pp 313–320, Academic Press, New York.
Eftink, M. R., & Biltonen, R. L. (1987) in *Hydrolytic Enzymes* (Neuberger, A., & Brocklehurst, K., Eds.) pp 333–376, Elsevier, New York.
Estell, D. A., Graycar, T. P., Miller, J. V., Powers, D. B., Burnier, J. P., Ng, P. G., & Wells, J. A. (1986) *Science 233*, 659–663.
Ferrin, T. E., Huang, C. C., Jarvis, L. E., & Langridge, R. (1988) *J. Mol. Graphics 6*, 13–27.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, Freeman, New York.
Fontecilla-Camps, J. C., de Llorens, R., Le Du, M. H., & Cuchillo, C. M. (1993) in *Ribonucleases: Chemistry, Biology, Biotechnology, 3rd International Meeting*, Abstract L6, GP srl Pubbliche Relazioni, Naples, Italy.
Ipata, P. L., & Felicioli, R. A. (1968) *FEBS Lett. 1*, 29–31.
Irie, M., Watanabe, H., Ohgi, K., Tobe, M., Matsumura, G., Arata, Y., Hirose, T., & Inayama, S. (1984) *J. Biochem. 95*, 751–759.
Jencks, W. P. (1987) *Catalysis in Chemistry and Enzymology*, Dover, Mineola, NY.
Karpeisky, M. Ya., & Yakovlev, G. I. (1981) *Sov. Sci. Rev., Sect. D 2*, 145–257.
Katoh, H., Yoshinaga, M., Yanagita, T., Ohgi, K., Irie, M., Beintema, J. J., & Meinsma, D. (1986) *Biochim. Biophys. Acta 873*, 367–371.
Kim, J.-S., & Raines, R. T. (1993a) *J. Biol. Chem. 268*, 17392–17396.
Kim, J.-S., & Raines, R. T. (1993b) *Protein Sci. 2*, 348–356.
Knowles, J. R. (1987) *Science 236*, 1252–1258.
Kornberg, A., & Baker, T. (1992) *DNA Replication*, Freeman, New York.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.
McGeehan, G. M., & Benner, S. A. (1989) *FEBS Lett. 247*, 55–56.
McPherson, A., Brayer, G., Cascio, D., & Williams, R. (1986) *Science 232*, 765–768.

Miranda, R. R. (1990) Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA.
Nossal, N. G., & Singer, M. F. (1968) *J. Biol. Chem. 243*, 913–922.
Ogilvie, K. K., Beaucage, S. L., Schifman, A. L., Theriault, N. Y., & Sadana, K. L. (1978) *Can. J. Chem. 56*, 2768–2780.
Parés, X., Nogués, M. V., de Llorens, R., & Cuchillo, C. M. (1991) *Essays Biochem. 26*, 89–103.
Raines, R. T., & Rutter, W. J. (1989) in *Structure and Chemistry of Ribonucleases, Proceedings of the First International Meeting* (Pavlovsky, A. G., & Polyakov, K., Eds.) pp 95–100, USSR Academy of Sciences, Moscow.
Reidhaar-Olson, J. F., Bowie, J. U., Breyer, R. M., Hu, J. C., Knight, K. L., Lim, W. A., Mossing, M. C., Parsell, D. A., Shoemaker, K. R., & Sauer, R. T. (1991) *Methods Enzymol. 208*, 564–586.
Rheinnecker, M., Baker, G., Eder, J., & Fersht, A. R. (1993) *Biochemistry 32*, 1199–1204.
Ribó, M., Fernández, E., Bravo, J., Osset, M., Fallon, M. J. M., de Llorens, R., & Cuchillo, C. M. (1991) in *Structure, Mechanism and Function of Ribonucleases* (de Llorens, R., Cuchillo, C. M., Nogués, M. V., & Parés, X., Eds.) pp 157–162, Universitat Autónoma de Barcelona, Bellaterra, Spain.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes 4*, 647–806.
Rushizky, G. W., Knight, C. A., & Sober, H. A. (1961) *J. Biol. Chem. 236*, 2732–2737.
Santoro, J., Gonźalez, C., Bruix, M., Neira, J. L., Neito, J. L., Herranz, J., & Rico, M. (1993) *J. Mol. Biol. 229*, 722–734.
Scrutton, N. S., Berry, A., & Perham, R. N. (1990) *Nature 343*, 38–43.
Sinha, N. D., Biernat, J., McManus, J., & Koster, H. (1984) *Nucleic Acids Res. 12*, 4539–4557.
Thompson, J. E., & Raines, R. T. (1994) *J. Am. Chem. Soc.* (in press).
Thompson, J. E., Venegas, F. D., & Raines, R. T. (1994) *Biochemistry* (in press).
Warshaw, M. M., & Tinoco, I. (1966) *J. Mol. Biol. 20*, 29–38.
Wells, J. A., Powers, D. B., Bott, R. B., Graycar, T. P., & Estell, D. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1219–1223.
Wilks, H. M., Hart, K. W., Feeney, F., Dunn, C. R., Muirhead, H., Chia, W. N., Barstow, D. A., Atkinson, T., Clarke, A. R., & Holbrook, J. J. (1988) *Science 24*, 1541–1544.
Wlodawer, A. (1985) in *Biological Macromolecules and Assemblies, Vol. II, Nucleic Acids and Interactive Proteins* (Jurnak, F. A., & McPherson, A., Eds.) pp 393–439, Wiley, New York.
Wlodawer, A., & Sjölin, L. (1983) *Biochemistry 22*, 2720–2728.
Yakovlev, G. I., Moiseyev, G. P., Bezborodova, S. I., Both, V., & Sevcik, J. (1992) *Eur. J. Biochem. 204*, 187–190.
Zuckermann, R. N., & Schultz, P. G. (1988) *J. Am. Chem. Soc. 110*, 6592–6594.