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A Residue to Residue Hydrogen Bond Mediates the Nucleotide Specificity of Ribonuclease A

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Bovine pancreatic ribonuclease A (RNase A) catalyzes the cleavage of the P-O$^{5'}$ bond of RNA after residues bound in the enzyme's B1 subsite. This subsite binds to cytidine 30-fold more tightly than to uridine and >10^5-fold more tightly than to adenine. Structural studies had suggested that the hydroxyl group of Thr45 can interact directly with the base of a bound nucleotide. In contrast, the carboxylate group of Asp83 cannot interact directly with bound substrate but can accept a hydrogen bond from the hydroxyl group of Thr45. To assess the role of the Thr45–Asp83 hydrogen bond in catalysis, T45G, D83A and T45G/D83A RNase A were prepared and their abilities to catalyze the cleavage of various substrates were determined. The results indicate that the side-chain of Asp83 enhances catalysis of reactions in which uridine is bound in the B1 subsite, but that this enhancement relies on the side-chain of Thr45. In contrast, the side-chain of Asp83 does not contribute to catalysis of reactions with cytidine in the B1 subsite. Thermodynamic cycles derived from kinetic parameters for the cleavage of poly(U) indicate that the Thr45-Asp83 interaction contributes 1.2 kcal/mol to transition state stabilization, which is 0.9 kcal/mol greater than its contribution to ground state stabilization. Thus, like many residue–substrate interactions, this residue to residue interaction enhances catalysis by becoming stronger as the reaction approaches the transition state.

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Introduction

Catalysis by enzymes is characterized by extreme efficiency and exquisite specificity. High-resolution structures determined by X-ray diffraction analysis and NMR spectroscopy have provided detailed pictures of the non-covalent interactions between enzymes and their substrates. The resulting insights, coupled with studies of substrate binding and turnover, have enabled enzymologists to discern the raison d'être of many enzymic residues. Still, a primary goal remains, the creation of new catalysts by the rational manipulation of enzyme-substrate interactions. Most efforts have focused on altering the substrate specificity of naturally occurring enzymes (Bone & Agard, 1991; Hedstrom, 1994). A major

obstacle is that many residues that mediate substrate specificity are not amenable to alteration because they are integral to substrate turnover or are involved in the maintenance of protein structure and stability (Knowles, 1987). Further, enzyme-substrate interactions are expected to be most favorable at the transition state of a chemical reaction. The *de novo* design of a structure that can turnover a substrate is thus no less difficult than predicting accurately the structure of the transition state. Previous efforts at enzyme redesign have involved exchanging entire domains (Hedstrom, 1994) or changing residues that interact directly with bound substrate (Bone & Agard, 1991). Here we report on how a hydrogen bond between two enzymic residues can influence substrate specificity.

Bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5) is a small protein (13.7 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; Eftink & Biltonen, 1987; Beintema *et al.*, 1988). Now that its gene is accessible to the techniques of recombinant DNA (Carsana *et al.*,
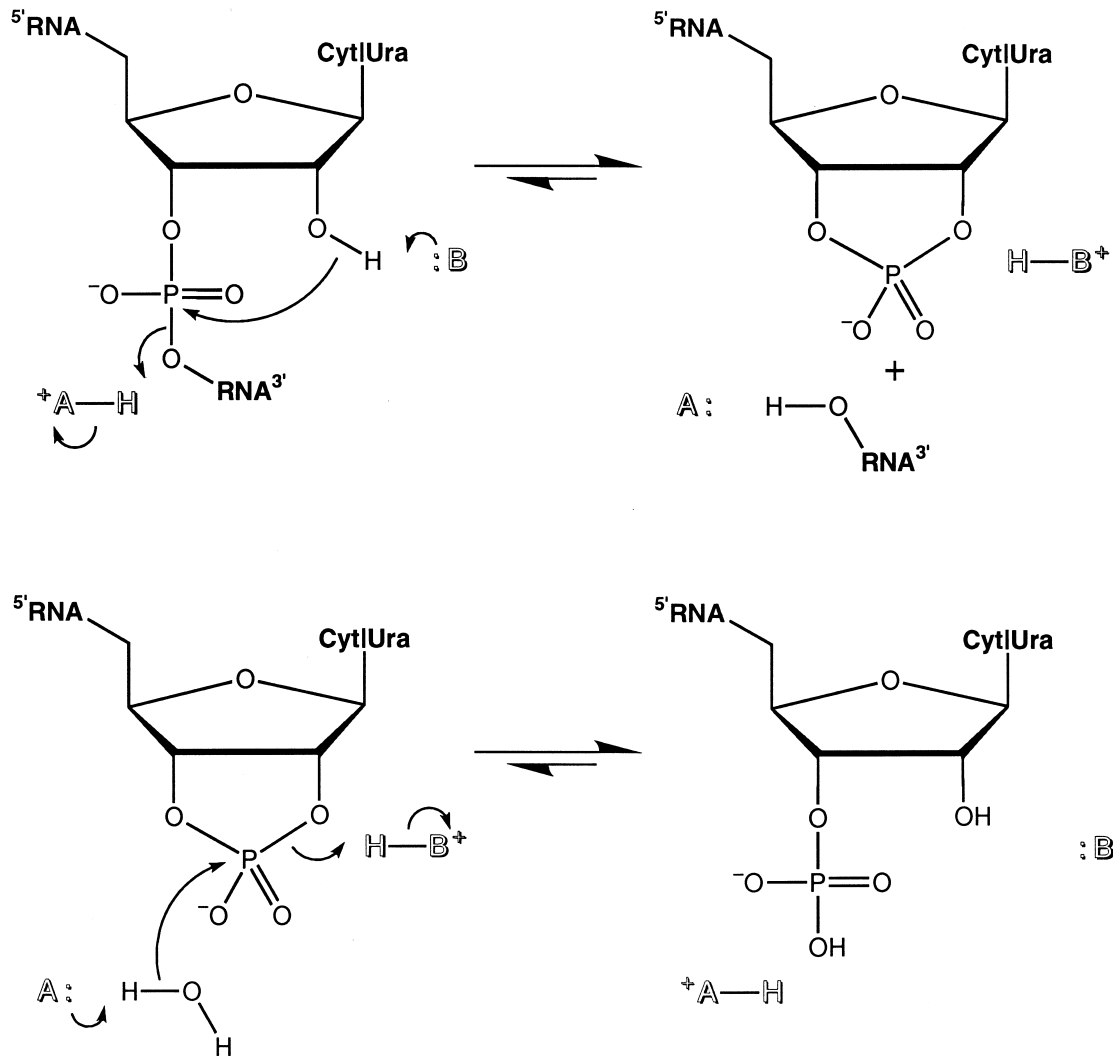
Figure 1. Mechanism of the transphosphorylation (top) and hydrolysis (bottom) reaction catalyzed by RNase A. B is His12; A is His119 (Thompson & Raines, 1994).

delCardayré *et al.*, 1995), this enzyme is poised to contribute still more.

RNase A binds the bases of adjacent RNA residues in three enzymic subsites, B1, B2 and B3 (Parés *et al.*, 1991), and catalyzes the cleavage of the P-O$^{5'}$ bond specifically on the 3'-side of nucleotides that are bound in the B1 subsite by the mechanism shown in Figure 1. The B1 subsite apparently binds only pyrimidine nucleotides (McPherson *et al.*, 1986; Aguilar *et al.*, 1992) and demonstrates an approximately 30-fold preference for cytidine *versus* uridine. The B2 and B3 subsites bind all residues but with a preference for those having a purine base (Rushizky *et al.*, 1961; Irie *et al.*, 1984; Fontecilla-Camps *et al.*, 1994).

The B1 subsite, which determines the primary specificity of RNase A, is formed by residues Thr45, Asp83, Phe120 and Ser123 (Figure 2; and see Wlodawer, 1985; Parés *et al.*, 1991). Each of these residues is conserved in at least 36 of the 41 known amino acid sequences of pancreatic ribonucleases (Beintema, 1987). The side-chain hydroxyl and main-chain carbonyl groups of Thr45 mediate the

pyrimidine specificity of RNase A by forming hydrogen bonds to a pyrimidine base (Figure 3; O$^{\gamma 1}$-N$_{(3)}$ distance 2.7 Å, O$^{\gamma 1}$-H-N$_{(3)}$ angle 147°; N-O$_{(2)}$ distance 2.6 Å, N-H-O$_{(2)}$ angle 147°) and by sterically excluding a purine base (delCardayré & Raines, 1994). The side-chain of Phe120 makes van der Waals contact with a pyrimidine base bound to the B1 subsite and to the side-chain of Thr45. Although Phe120 may enhance the binding of bases to the B1 subsite, it does not appear to mediate purine/pyrimidine specificity (delCardayré & Raines, 1994). The side-chain of Asp83 accepts a hydrogen bond from the side-chain of Thr45 (O$^{\gamma 1}$-O$^{\delta 2}$ distance 2.7 Å, O$^{\gamma 1}$-H-O$^{\delta 2}$ angle 157°) when uridine, but not cytidine, is bound to the B1 subsite and may mediate the cytidine/uridine specificity of RNase A. The side-chain of Ser123 has been assumed to form a hydrogen bond to a uridine but not a cytidine residue bound to the B1 subsite, and may enhance the rate of cleavage after uridine residues (Hodges & Merryfield, 1975; Bruenger *et al.*, 1985). Such a hydrogen bond, however, is not evident in the combined X-ray/neutron diffraction structure of the RNase A
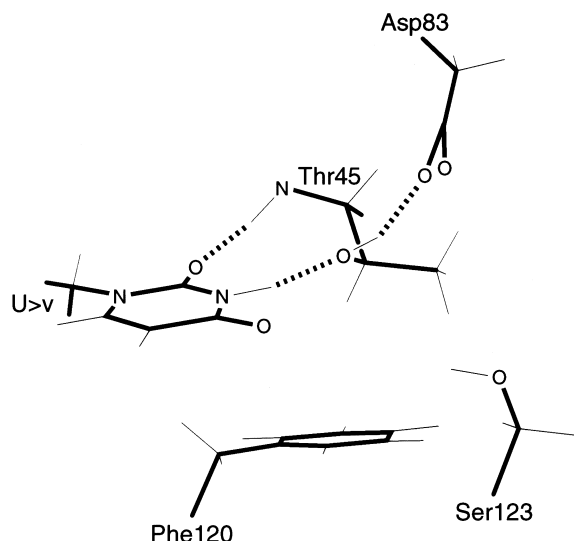
Figure 2. Structure of the B1 subsite in the complex of RNase A with uridine 2',3'-cyclic vanadate (U>v), a transition state analog (Wlodawer *et al.*, 1983). The structure was refined at 2.0 Å from X-ray (*R*-factor 0.191) and neutron (*R*-factor 0.207) diffraction data. Hydrogen bonds of < 3.0 Å are indicated by broken lines. Only the uracil base of U>v is shown.

complex with U>v (Wlodawer, 1985). Further, mutation of the analogous serine in angiogenin, a homolog of RNase A, has no effect on substrate specificity (Curran *et al.*, 1993).

Recently, we showed that replacing Thr45 with a glycine residue results in a 10^5-fold increase in the purine/pyrimidine specificity of RNase A for homopolymeric substrates (delCardayré & Raines, 1994). The T45G enzyme, however, retains the wild-type preference for poly(C) over poly(U). To investigate how Asp83 and its interaction with Thr45 affect the specificity of RNase A, we have created the mutants D83A and T45G/D83A and compared the substrate specificities of these enzymes with those of the wild-type and T45G enzymes. Our results indicate that the side-chain of Asp83 has no effect on the kinetics of cleavage after cytidine residues, but does affect significantly the rate of cleavage of poly(U) and hydrolysis of U>p through an interaction that is dependent on the side-chain of Thr45. Apparently, the Thr45-Asp83 hydrogen bond increases the ability of RNase A to

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† The role of the active-site threonine residue in mediating this preference in homologs of RNase A may be more pronounced (Miranda, 1990; Curran *et al.*, 1993).

‡ This analysis assumes that $K_m = K_s$. The value of k_{cat}/K_m for the cleavage of poly(U) by wild-type RNase A is 4×10^5 M^{-1} s^{-1} (Table 1), which is significantly lower than that expected for a reaction limited by diffusion (Blacklow *et al.*, 1988). Hence, the values of K_m and k_{cat}/K_m report on the ability of RNase A to bind to the ground state and transition state, respectively, for the transphosphorylation reaction.

cleave uridine-containing substrates by the selective stabilization of the transition state for this reaction. In addition, the side-chain of Asp83 enhances the rate of cleavage of poly(A), but through an interaction that is not dependent on the side-chain of Thr45. These results indicate that, like a direct interaction between an enzyme and its substrate, an interaction between two functional groups within an enzyme can contribute to substrate specificity.

Results

Steady-state kinetic parameters

Steady-state kinetic parameters for the cleavage of poly(U), poly(C) and poly(A), and for the hydrolysis of U>p and C>p by wild-type, T45G, D83A and T45G/D83A RNase A are shown in Table 1. Also shown are the values of t_m, which indicate that the kinetic parameters determined at 25°C are indeed those of native protein. As reported previously (delCardayré & Raines, 1994), replacing the side-chain of residue 45 with a glycine residue caused the value of k_{cat}/K_m for cleavage of poly(A) to increase. This increase was a result of both an increase in k_{cat} and a decrease in K_m. The T45G enzyme also displayed an increased ability to catalyze the cleavage of poly(I) (data not shown). In contrast, the relative preference of the wild-type enzyme for poly(U) and poly(C) was maintained in the T45G mutant (delCardayré & Raines, 1994). This preference is therefore not mediated by Thr45†.

The kinetic consequences of mutating Asp83 were less dramatic than were those of mutating Thr45 (Table 1). D83A RNase A displayed a decrease in k_{cat}/K_m for the cleavage of poly(U) and poly(A), and the hydrolysis of U>p as compared with wild-type RNase A. As shown in Figure 4, the decrease in k_{cat}/K_m for the cleavage of poly(U) depended on the side-chain of residue 45. In contrast, the decrease in k_{cat}/K_m for the cleavage of poly(A) was independent of the side-chain of residue 45. The D83A enzyme cleaved poly(C) and hydrolyzed C>p with kinetic parameters similar to those of wild-type RNase A (Table 1). Changing Asp83 to alanine did, however, cause an increase in k_{cat}/K_m for the cleavage of poly(C) by T45G RNase A (Figure 4).

Thermodynamic cycles

Thermodynamic cycles that relate the free energies corresponding to k_{cat}, K_m and k_{cat}/K_m for the cleavage of poly(U) by wild-type, D83A, T45G and T45G/D83A RNase A are shown in Figure 5. The effect of a mutation on the free energy of binding the transition state and ground state can be determined from the k_{cat}/K_m and $1/K_m$ boxes, respectively‡. The side-chain of Thr45 contributes 2.7 kcal/mol to the
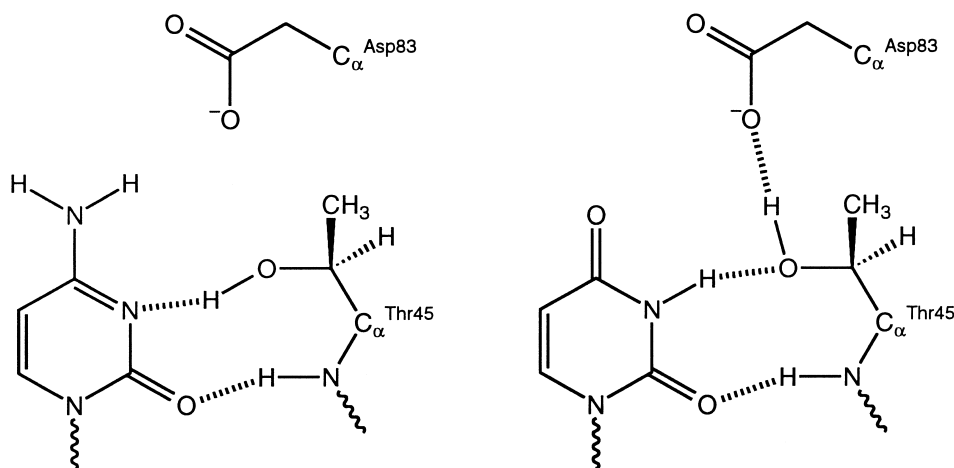
Figure 3. Hydrogen bonds formed between a bound cytidine (left) or uridine (right) nucleotide and the residues of the B1 subsite of RNase A (W. A. Gilbert, A. L. Fink & G. A. Petsko, unpublished results; Wlodawer *et al.*, 1983).

binding of the transition state and 0.7 kcal/mol to the binding of the ground state. The side-chain of Asp83 is less important, contributing 1.4 and 0.6 kcal/mol to transition state and ground state binding, respectively. Changing Asp83 to alanine diminishes the contribution of Thr45 to the binding of the transition state (now 1.4 from 2.7 kcal/mol) or ground state (now 0.3 from 0.7 kcal/mol). Similarly, changing Thr45 to glycine almost eliminates the contribution of Asp83 to the binding of the transition state (now 0.2 from 1.4 kcal/mol) or ground state (now 0.3 from 0.6 kcal/mol). Finally, the contributions to the free energy of binding the transition state and ground state due to the Thr45-Asp83 interaction were −1.2 kcal/mol and −0.3 kcal/mol, respectively.

Molecular modeling

Models of T45A and T45G RNase A, created by using the SWAP function of the MIDAS molecular modeling program (Ferrin *et al.*, 1988), suggested that truncating the side-chain of Thr45 creates a cavity that could accommodate an adenine residue. In these models, the carboxyl group of Asp83 is within hydrogen-bonding distance of the exocyclic amino group of an adenine but not a cytidine residue bound in the B1 subsite of T45G RNase A.

Discussion

Most biochemical phenomena can be reduced to a problem of molecular recognition, and catalysis by

Table 1. Steady-state kinetic parameters for the cleavage of poly(U), poly(C) and poly(A), and hydrolysis of U>p and C>p by wild-type, T45G, D83A and T45G/D83A RNase A

RNase A (t_m, °C)	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (10^6 M^{-1} s^{-1})
Wild-type	Poly(U)	24 ± 1	0.06 ± 0.01	0.40 ± 0.05
(63)	Poly(C)	510 ± 10	0.034 ± 0.002	15 ± 1
	Poly(A)	0.023 ± 0.001	0.080 ± 0.009	0.00028 ± 0.00001
	C>p	3.7 ± 0.7	1.0 ± 0.6	0.004 ± 0.002
	U>p	2.9 ± 0.4	3.2 ± 0.5	0.00093 ± 0.00006
T45G	Poly(U)	0.86 ± 0.08	0.19 ± 0.04	0.0045 ± 0.0004
(53)	Poly(C)	1000 ± 300	4 ± 2	0.24 ± 0.07
	Poly(A)	5.8 ± 0.2	0.023 ± 0.004	0.25 ± 0.05
	C>p	0.5 ± 0.2	7 ± 2	0.000070 ± 0.000010
	U>p	0.17 ± 0.09	30 ± 10	0.0000067 ± 0.0000005
D83A	Poly(U)	6 ± 1	0.18 ± 0.09	0.037 ± 0.007
(59)	Poly(C)	240 ± 10	0.022 ± 0.004	11 ± 2
	Poly(A)	0.017 ± 0.003	0.23 ± 0.07	0.000069 ± 0.000006
	C>p	2.5 ± 0.2	1.2 ± 0.3	0.0023 ± 0.0005
	U>p	1.0 ± 0.3	11 ± 3	0.000094 ± 0.000002
T45G/	Poly(U)	1.0 ± 0.4	0.3 ± 0.2	0.0033 ± 0.0008
D83A	Poly(C)	380 ± 50	0.18 ± 0.05	2.1 ± 0.3
(47)	Poly(A)	3.5 ± 0.1	0.034 ± 0.007	0.11 ± 0.01

Data for the cleavage of polymeric substrates by wild-type and T45G RNase A has been reported previously (delCardayré & Raines, 1994). Values of t_m are reported ±2 deg.C.
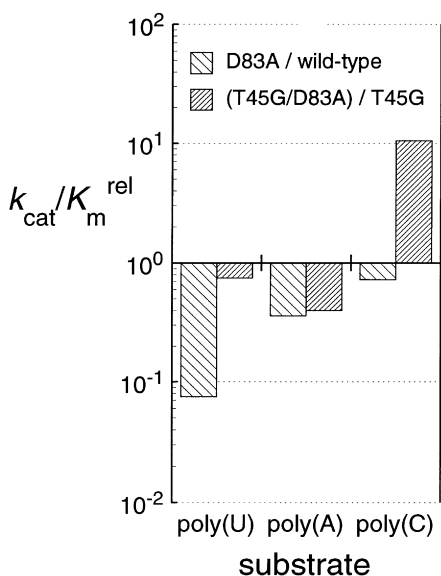
Figure 4. The effect of Asp83 on the specificity of RNase A. Bars indicate the effect of replacing Asp83 with an alanine residue in wild-type or T45G RNase A on the specificity constant for cleavage of homopolymeric substrate.

enzymes is not an exception. Enzymes have evolved to be complementary in structure to the transition state of the reactions that they catalyze (Haldane, 1930). This complementarity is so precise that an enzyme can stabilize the transition state of a reaction by up to 32 kcal/mol relative to that of the un-catalyzed reaction, resulting in rate enhancements of up to 10^{17}-fold (Radzicka & Wolfenden, 1995). Still, enzymes exhibit only modest affinity for the ground state of substrates and products. Apparently, during catalysis the interactions between an enzyme and substrate become increasingly favorable as the reaction proceeds. Substrate specificity is therefore determined primarily by the extent to which enzyme-substrate interactions can mature as the reaction approaches the transition state.

Recently, we reported that the side-chain of a residue that interacts directly with bound substrate (Thr45; Figure 2) is responsible for the purine/pyrimidine specificity of RNase A (delCardayré &

Raines, 1994). Thr45 is a surface residue that is relatively remote from the general base (His12) and general acid (His119) that expedite cleavage of the P-O$^{5'}$ bond of RNA (Figure 1). A mutant RNase A in which Thr45 is replaced with a glycine residue displays a 10^5-fold change in purine/pyrimidine specificity. This change results from a 10^3-fold increase in the specificity constant (k_{cat}/K_m; Fersht, 1985) for the cleavage of poly(A), and a 10^2-fold decrease in that for the cleavage of poly(C) and poly(U) (Table 1). As can be seen in Table 1, the side-chain of Thr45 affects k_{cat}/K_m considerably more that it does K_m for the cleavage of all substrates but poly(C), demonstrating the importance of this residue in substrate specificity.

In addition to Thr45, another solvent-accessible residue that contributes to the B1 subsite of RNase A is Asp83 (Figure 2). In contrast to Thr45, Asp83 appears to have no direct interaction with a pyrimidine residue bound to the B1 subsite. Rather, the carboxylate group of Asp83 appears to accept a hydrogen bond from the hydroxyl group of Thr45 when uridine but not cytidine is bound to the B1 subsite of RNase A (Figure 3). Our results indicate that this interaction does indeed exist and demonstrate its contribution to the substrate specificity of RNase A.

Replacing Asp83 with an alanine residue has no significant effect on the cleavage of poly(C) or the hydrolysis of C>p by wild-type RNase A (Figure 4; Table 1). In contrast, this mutation decreases the ability of wild-type (but not T45G) RNase A to bind and turnover both poly(U) and U>p. Thus, the influence of the side-chain of Asp83 on the specificity of RNase A is dependent on the side-chain of Thr45. To probe further the interaction between Thr45 and Asp83, the cleavage of poly(U) by wild-type, T45G, D83A and T45G/D83A RNase A was analyzed by using thermodynamic cycles (Figure 5). This analysis indicates that the Thr45-Asp83 interaction contributes 0.3 kcal/mol to stabilizing the ground state and 1.2 kcal/mol to stabilizing the transition state during the cleavage of poly(U). Thus, the interaction between Thr45 and Asp83 effects the specificity of RNase A by contributing to the selective stabilization of the transition state by 0.9 kcal/mol. This stabilization is likely to result from the
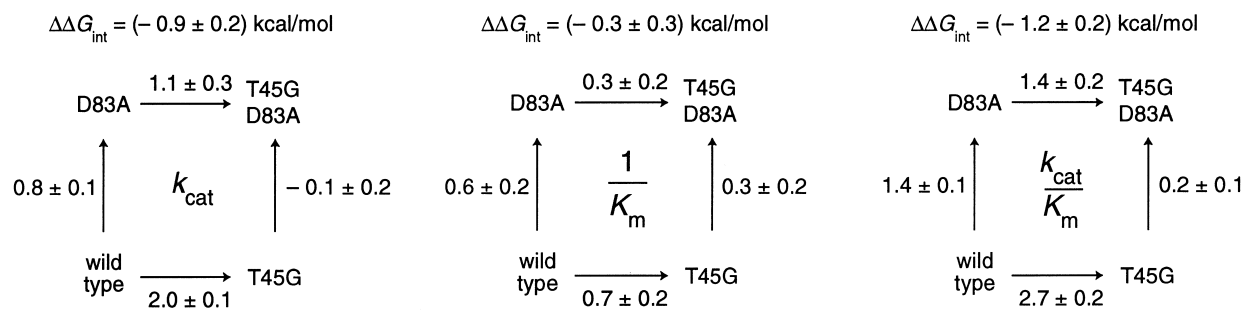

Figure 5. Thermodynamic cycles for the cleavage of poly(U) by RNase A upon mutation of Thr45 and Asp83. The cycles are drawn for the steady-state kinetic parameters k_{cat} (left), $1/K_m$ (middle) and k_{cat}/K_m (right) with values (in kcal/mol) derived from equations analogous to equation (2). $\Delta\Delta G_{int}$ is the free energy of interactions between the side-chains of Thr45 and Asp83 for each kinetic parameter, and is derived from equation (3).

strengthening of the Thr45-Asp83 hydrogen bond, which would make the hydroxyl oxygen electron-deficient and hence a better hydrogen bond acceptor. This model is consistent with differences observed in crystalline complexes of RNase A bound to mimics of the ground state and transition state. The distance between $O^{\gamma 1}$ of Thr45 and $O^{\delta 2}$ of Asp83 in RNase A alone (4.2 Å; Wlodawer *et al.*, 1986) or complexed to inorganic phosphate (3.6 Å; Wlodawer *et al.*, 1988) is significantly great than that in RNase A complexed to the transition state analog U>v (2.7 Å; Figure 2; Wlodawer *et al.*, 1983)†.

The existence of a hydrogen bond between Thr45 and Asp83 also explains why the chemical shift of the resonance of Asp83 changes upon binding cytidine 3'-phosphate but not upon binding uridine 3'-phosphate (Bruix *et al.*, 1991). In the unliganded enzyme, Thr45 and Asp83 apparently share a hydrogen bond. When uridine 3'-phosphate binds to the enzyme this bond is maintained; but when cytidine 3'-phosphate binds, it is lost.

The hydroxyl group of Thr45 is versatile. Rotation of the C^{β}-$O^{\gamma 1}$ bond can present either a hydrogen bond to cytidine or a lone pair of electrons to uridine (Figure 3). When uridine is bound, the Thr45 hydroxyl group can also donate a hydrogen bond to the carboxyl group of Asp83. This hydrogen bond serves to align the side-chain of Thr45. In its absence, the side-chain of Thr45 would have to overcome additional rotational entropy to bind uridine. The change in entropy upon restriction of rotation about a single bond has been estimated (Page & Jencks, 1971), to be $\Delta S° = -4.5$ cal/(mol K), which corresponds to $T\Delta S = -1.3$ kcal/mol at 25°C. More specifically, the entropy lost by a threonine residue upon forming a hydrogen bond with another residue has been estimated to be $T\Delta S = -1.6$ kcal/mol at 25°C (Pickett & Sternberg, 1993). These estimated entropic costs are similar to the observed change in the free energy of the transition state for poly(U) cleavage that is imparted by mutation of Asp83 to an alanine residue ($\Delta\Delta G = -1.4$ kcal/mol; Figure 5).

The side-chain of Asp83 also contributes to poly(C) binding in T45G RNase A (Figure 4). Molecular modeling suggests that the carboxyl group of Asp83 cannot interact directly with a cytidine residue bound to the B1 subsite of wild-type RNase A. Perhaps, in the T45G enzyme, cytidine could slide into the enlarged B1 subsite, where its exocyclic amino group could donate a hydrogen bond to the carboxyl side-chain of Asp83. Such an interaction might interfere with the proper alignment of His12 and His119 with a cytidine residue (Figure 1). By removing the side-chain of Asp83, such an undesirable interaction would be removed and proper alignment restored.

Finally, the side-chain of Asp83 alters, to some extent, the interaction of RNase A with poly(A). The value of k_{cat}/K_m for the cleavage of poly(A) is decreased by fourfold upon mutation of Asp83 to an alanine residue in either the wild-type or T46G enzyme. Molecular modeling suggests that this effect could arise from a hydrogen bond between the exocyclic amino group of adenine and the carboxyl group of Asp83.

Ribonuclease A digests the RNA produced by stomach microflora, presumably to salvage phosphorus and nitrogen (Barnard, 1969; Jermann *et al.*, 1995). Thus, it seems likely that the enzyme would have evolved to be non-specific. Previously, we identified two point mutations that broaden the specificity of RNase A by allowing for the efficient cleavage of RNA after adenine residues, thereby increasing the number of cleavage sites in an RNA strand by 50% (delCardayré & Raines, 1995). These mutations are not prudent, however, as they decrease by 10^1 to 10^2-fold the values of k_{cat}/K_m for pyrimidine residues (delCardayré & Raines, 1994). Here, we have demonstrated that retaining the side-chain of Asp83 also makes RNase A less specific, but without impairing its catalytic prowess.

Conclusion

Thr45 and Asp83 are in the B1 subsite, removed from the site in RNase A where covalent bonds are made and broken. Thr45 interacts directly with bound substrate while Asp83 interacts directly with the side-chain of Thr45 but not with bound substrate. Each of these residues mediates substrate specificity and does so by enhancing the ability of RNase A to bind to the rate-limiting transition state during catalysis: both the interaction of Thr45 and bound substrate and that between Thr45 and Asp83 provide maximal binding energy at the transition state. These findings demonstrate how a residue that is not in direct contact with substrate can influence the substrate specificity of an enzyme and demonstrate how the specificity of RNase A is manifested during transition state rather than ground state binding.

Materials and Methods

Materials

Escherichia coli strain BL21(DE3) (F⁻ompT r_B⁻m_B⁻; Studier & Moffat, 1986) was from Novagen (Madison, WI). *E. coli* strain CJ236 was from Bio-Rad (Richmond, CA). All enzymes for molecular biology were from Promega (Madison, WI). Reagents for DNA synthesis were from Applied Biosystems (Foster City, CA), except for acetonitrile, which was from Baxter Healthcare (McGaw Park, IL). Other ribonuclease substrates were obtained from Sigma Chemical (St Louis, MO). Commercial

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† Interpreting the results of mutagenesis experiments can be complicated by fortuitous changes in a protein's structure that are deleterious to its function (Knowles, 1987). For the following reasons, we believe that truncating the side-chain of Thr45 and Asp83 did not result in any such changes. First, these residues are accessible to solvent. Second, all kinetic parameters were determined at a temperature >20 deg.C lower than the t_m of the enzyme. Finally, the value of k_{cat} for the cleavage of poly(C) by the wild-type and each mutant enzyme is similar (Table 1).

polyribonucleic acids were purified by precipitation in ethanol in the presence of ammonium acetate, and had an average length of approximately 200 bp, as determined by polyacrylamide gel electrophoresis. All other chemicals were obtained as described (delCardayré & Raines, 1994) and used without further purification.

General methods

DNA oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer, and purified by Oligonucleotide Purification Cartridges (Applied Biosystems). DNA sequences were determined with a Sequenase Version 2.0 kit from United States Biochemicals (Cleveland, OH).

Ultraviolet and visible absorbance measurements were made with a Cary 3 spectrophotometer equipped with a Cary temperature controller. RNase A concentrations were determined by assuming that $\epsilon_{1cm}^{0.1\%} = 0.72$ at 277.5 nm (Sela *et al.*, 1957). pH was measured with a Beckman pH meter fitted with a Corning electrode, calibrated at room temperature with standard buffers from Fischer (Chicago, IL).

Mutagenesis

Site-directed mutagenesis (Kunkel *et al.*, 1987) was performed on a single-stranded DNA isolated from *E. coli* strain CJ236. The codon for Asp83 in plasmids pBXR1 and pBXR1.T45G (delCardayré & Raines, 1994) was replaced with that for an alanine residue by using oligonucleotide SD42 (GGTCTCACGGCACGCGGTGATGCTCAT). The resulting plasmids were transported into *E. coli* BL21(DE3). D83A and T45G/D83A RNase A were produced and purified to homogeneity by using procedures described previously (delCardayré *et al.*, 1995).

Steady-state kinetic analyses

The cleavage of poly(U), poly(C) and poly(A), and the hydrolysis of U>p and C>p were monitored by UV spectroscopy. The $\Delta\epsilon$ for these reactions, calculated from the difference in molar absorptivity of the polymeric substrate and the mononucleotide cyclic phosphate product or of the nucleotide 2',3'-cyclic phosphate substrate and the nucleotide 3'-phosphate product, was $1360\ M^{-1}\ cm^{-1}$ for poly(U) at 278 nm, $2380\ M^{-1}\ cm^{-1}$ for poly(C) at 250 nm, $6400\ M^{-1}\ cm^{-1}$ for poly(A) at 260 nm, $600\ M^{-1}\ cm^{-1}$ for U>p at 286 nm, and $1450\ M^{-1}\ s^{-1}$ for C>p at 287 nm. Assays were performed at 25°C in 0.10 M Mes-HCl buffer (pH 6.0) containing 0.10 M NaCl, 10 μM to 4.0 mM substrate, and 1.0 nM to 1.0 μM enzyme. The values for k_{cat}, K_m and k_{cat}/K_m were determined from initial velocity data with the program HYPERO (Cleland, 1979).

Thermal stability

As RNase A is denatured, its six tyrosine residues become exposed to solvent and its extinction coefficient at 287 nm decreases significantly (Hermans & Scheraga, 1961). The thermal stabilities of wild-type, T45G, D83A and T45G/D83A RNase A were assessed by monitoring the change in A_{278} with temperature, as described by Pace *et al.* (1989). Briefly, the temperature of a solution of protein (0.1 to 0.5 mg/ml) in 0.10 M Mes-HCl buffer (pH 6.0) containing 0.10 M NaCl was increased in 0.2 deg.C increments between 20 and 85°C, and A_{278} was recorded

after three minutes of equilibration at each new temperature. The data were fit to a two state model for denaturation, and t_m (the midpoint in the thermal denaturation curve) was calculated.

Thermodynamic cycles

Thermodynamic cycles were calculated from values of k_{cat}, K_m and k_{cat}/K_m for the cleavage of poly(U) by wild-type, T45G, D83A and T45G/D83A RNase A. The change in the contribution of free energy to catalysis ($\Delta\Delta G$) due to a particular mutation was calculated from equation (1), where *f* was the ratio of a particular kinetic parameter for the two enzymes being compared:

$$\Delta\Delta G = RT \ln f \qquad (1)$$

For example, the contribution of free energy to stabilizing the transition state for poly(U) cleavage by the side-chain of Asp83 was calculated from equation (2):

$$\Delta\Delta G_{wt \to D83A} = RT \ln[(k_{cat}/K_m)_{wt}/(k_{cat}/K_m)_{D83A}] \qquad (2)$$

The contribution of free energy to catalysis from the interaction between the two residues under investigation was calculated from equation (3) as described (Carter *et al.*, 1984; Horowitz & Fersht, 1990; Horowitz *et al.*, 1990; Mildvan *et al.*, 1992).

$$\Delta\Delta G_{int} = \Delta\Delta G_{wt \to T45G/D83A} - \Delta\Delta G_{wt \to T45G} - \Delta\Delta G_{wt \to D83A} \qquad (3)$$

Acknowledgements

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