Structural investigation of catalytically modified F120L and F120Y semisynthetic ribonucleases

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Abstract

The structures of two catalytically modified semisynthetic RNases obtained by replacing phenylalanine 120 with leucine and tyrosine have been determined and refined at a resolution of 2.0 Å ($R = 0.161$ and 0.184 , respectively). These structures have been compared with the refined 1.8-Å structure ($R = 0.204$) of the fully active phenylalanine-containing enzyme (Martin PD, Doscher MS, Edwards BFP, 1987, *J Biol Chem* 262:15930–15938) and with the catalytically defective D121A (2.0 Å, $R = 0.172$) and D121N (2.0 Å, $R = 0.186$) analogs (deMel VSJ, Martin PD, Doscher MS, Edwards BFP, 1992, *J Biol Chem* 267:247–256). The movement away from the active site of the loop containing residues 65–72 is seen in all three catalytically defective analogs—F120L, D121A, and D121N—but not in the fully active (or hyperactive) F120Y. The insertion of the phenolic hydroxyl of Tyr 120 into a hydrogen-bonding network involving the hydroxyl group of Ser 123 and a water molecule in F120Y is the likely basis for the hyperactivity toward uridine 2',3'-cyclic phosphate previously found for this analog (Hodges RS, Merrifield RB, 1974, *Int J Pept Protein Res* 6:397–405) as well as the threefold increase in K_M for cytidine 2',3'-cyclic phosphate found for this analog by ourselves.

Keywords: comparison of active site structure; loop conformational change; modulation of hydrogen bonding; mutants; protein semisynthesis; semisynthetic RNases; X-ray structure

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Abbreviations: RNase A, bovine pancreatic ribonuclease A; RNase S, RNase A in which the 20–21 peptide bond has been hydrolyzed by the action of subtilisin; RNase 1–118, polypeptide consisting of residues 1–118 of RNase A; RNase 111–124, tetradecapeptide consisting of residues 111–124 of RNase A; RNase 111–124(F120L), RNase 111–124 in which Phe 120 has been substituted by leucine; RNase 111–124(F120Y), RNase 111–124 in which Phe 120 has been substituted by tyrosine; RNase 111–124(D121A), RNase 111–124 in which Asp 121 has been substituted by alanine; RNase 111–124(D121N), RNase 111–124 in which Asp 121 has been substituted by asparagine; RNase 111–124(S123A), RNase 111–124 in which Ser 123 has been substituted by alanine; RNase 1–118:111–124, noncovalent complex of RNase 1–118 and RNase 111–124, also referred to as the “parent complex”; F120L, noncovalent complex of RNase 1–118 and RNase 111–124(F120L), also referred to as the “leucine analog”; F120Y, noncovalent complex of RNase 1–118 and RNase 111–124(F120Y), also referred to as the “tyrosine analog”; D121A, noncovalent complex of RNase 1–118 and RNase 111–124(D121A), also referred to as the “alanine analog”; D121N, noncovalent complex of RNase 1–118 and RNase 111–124(D121N), also referred to as the “asparagine analog”; S123A, noncovalent complex of RNase 1–118 and RNase 111–124(S123A).

The replacement of Phe 120 by leucine in the fully active semisynthetic RNase 1–118:111–124 (Fig. 1) (Lin et al., 1970) reduces k_{cat} to 13% (Lin et al., 1972; Table 1), confirming the functional role for this residue that was implied by its (then) invariant status (Beintema et al., 1988) as well as its propinquity to the confirmed active site residue, His 119. Conversely, the subsequent replacement of Phe 120 by tyrosine in RNase 1–118:111–124 resulted in an enzyme with full activity toward RNA and cytidine 2',3'-cyclic phosphate and enhanced activity toward uridine 2',3'-cyclic phosphate (Hodges & Merrifield, 1974). In order to understand more completely the way in which the structure at position 120 modulates catalytic efficiency in this enzyme, we have now determined the 2.0-Å structures of both F120L ($R = 0.161$) and F120Y ($R = 0.184$) using crystals grown under conditions identical to those used to crystallize RNase 1–118:111–124 (Doscher et al., 1983a). Crystals of both analogs proved to be in the same space group and to have very nearly the same unit cell dimensions as the fully active parent complex (Martin et al., 1987; Table 2). We report here on the structural changes observed in the two analogs, compare these changes

Fig. 1. Preparation and assembly of RNase 1-118:111-124. The schematic diagram shows the preparation of RNase 1-118 by limited proteolytic digestion and its assembly with a chemically synthesized 111-124 tetradecapeptide into a noncovalent complex (which is fully active when no amino acid replacements have been made).

Table 1. Kinetic parameters for cytidine 2',3'-cyclic phosphate at pH 6.0

Species	K_M (mM)	k_{cat} (s^{-1})	Reference ^a
RNase A ^b	1.66 ± 0.09	7.6 ± 0.4	1
RNase A ^c	0.30	1.7	2
RNase A ^d	2.6 ± 0.3	2.1 ± 0.2	3
RNase 1-118:111-124 ^e	0.7	98% ^f	4
RNase 1-118:111-124 ^g	1.1 ± 1.0	2.1 ± 0.8	3
(D121N) ^h	4.3 ± 0.6	0.24 ± 0.02	3
(D121A) ⁱ	1.9 ± 0.8	0.47 ± 0.09	3
(F120L) ^j	1.0	13% ^k	4
(F120Y) ^l	3.1 ± 0.3	4.0 ± 0.4^m	

^a References: ¹Herries et al., 1962; ²Ronda et al., 1976; ³deMel et al., 1992; ⁴Lin et al., 1972.

^b Assay conditions: 0.20 M KCl, pH maintained at 6.00 with a pH-stat, 25 °C, 0.92–8.8 mM substrate, RNase concentration not given.

^c Assay conditions: 0.1 M NaCl, 0.1 M sodium acetate, pH 6.0, 25 °C, 0.1–4 mM substrate, RNase concentration not given.

^d Assay conditions: 0.2 M NaCl, 0.1 M sodium malonate, pH 6.00, 25 °C, 0.58–6.0 mM substrate, 0.19 μ M RNase. Velocity determinations were made in quadruplicate and standard error values are given.

^e Assay conditions: 0.1 M NaCl, 0.1 M Tris acetate, pH 6.0, 25 °C, 0.1–0.9 mM substrate, RNase 1-118 concentration, 0.36 μ M.

^f No k_{cat} value was given, but the activity at saturating ratios of RNase 111-124 to RNase 1-118 was stated to be 98% of that for an equimolar amount of RNase A.

^g Buffer, temperature, and concentration range of substrate were the same as in footnote d; RNase 1-118 was 0.28 μ M. The velocity determinations were made in quadruplicate at four progressively higher mole ratios of RNase 111-124 to RNase 1-118, and the maximal velocity at saturating levels of peptide was determined by extrapolation of a double reciprocal plot of velocity versus mole ratio.

^h As in footnote g, except that RNase 1-118 was 3.36 μ M.

ⁱ As in footnote g, except that RNase 1-118 was 1.68 μ M.

^j Assay conditions as in footnote e, except that RNase 1-118 was 5 μ M.

^k No k_{cat} value was given, but the activity at saturating ratios of RNase 111-124(F120L) to RNase 1-118 was stated to be 13% of that for an equimolar amount of RNase A.

^l As in footnote g, except that the peptide was RNase 111-124(F120Y).

^m Hodges and Merrifield (1974) reported an activity of 97% for RNase 1-118:RNase 111-124(F120Y) at pH 6.0 and a substrate concentration of 0.5 mM. Our kinetic parameters predict an activity of 85% under these conditions.

with those seen previously in two catalytically defective analogs prepared by replacing Asp 121 in RNase 1-118:111-124 with asparagine and alanine, respectively (deMel et al., 1992), and discuss possible structural bases for the observed differences in activity in the residue 120 analogs.

Results

Data collection, structure determination, and refinement

The diffraction data and final refinement statistics for RNase 1-118:111-124 and the F120L and F120Y analogs are summarized in Table 2.

Crystals of both analogs belong to the same space group as the parent molecule, and their unit cell dimensions are nearly identical (Table 2). Initially, in each case, several cycles of rigid body refinement using ROTLSQ software (Hendrickson & Konnert, 1979) were run to find the orientation and position of

Table 2. Data collection and refinement

Statistics	RNase 1-118:111-124 ^a	F120Y	F120L
Unit cell (Å) ^b	$a = 67.68$ $c = 65.03$	$a = 68.15$ $c = 65.15$	$a = 67.25$ $c = 64.90$
Reflections used	12,117	11,776	9,078
Percent of total data	89.5	94.6	80.8
Resolution	10–1.8	5–2.0	5–2.0
$R_{sym}(I)$	0.116	0.062	0.065
R after ROTLSQ	0.360	0.284	0.271
R after X-PLOR coolstage	—	0.245	0.237
R after PROLSQ	0.204	0.193	0.173
R after X-PLOR B refinement	—	0.184	0.161
RMS bonds	0.025	0.025	0.025
RMS ω angle	1.6	1.9	1.7

^a From Martin et al. (1987).

^b All structures belong to space group P3₂21 and have one molecule per asymmetric unit.

the molecule. Application of the X-PLOR package of programs (Brünger, 1988) then lowered the *R*-factors to 0.237 for F120L and to 0.245 for F120Y. The refinements were continued using PROLSQ (Hendrickson & Konnert, 1979) and PROFFT (Agarwal, 1978; Finzel, 1987) programs, and adding water molecules, as well as a sulfate ion at the active site, until convergence was reached (Table 2). The final *R*-factors are 0.161 for F120L and 0.184 for F120Y.¹

The RMS difference between the C_{α} atoms of RNase 1–118:111–124 and those in the F120L analog is 0.37 Å, a value that indicates that the overall structure of the molecule has been left virtually unchanged by the phenylalanine to leucine substitution. Yet, inspection of the difference distance matrix (Nishikawa et al., 1972; Kundrot & Richards, 1987) for this analog reveals that several significant, localized changes in structure have occurred (Fig. 2; Kinemage 1). In contrast, the minimal structural changes indicated by the RMS difference for the C_{α} atoms for the Tyr 120 analog (0.29 Å) are confirmed by its difference distance matrix (Fig. 2; Kinemages 2, 3). The patterns of C_{α} displacements (Fig. 3) and temperature factors (Fig. 4) are similar in both analogs. Significant movements of 10 or so surface side chains are clearly visible (Fig. 3), but the pattern is very similar for F120L and F120Y. Certainly there is no outstanding difference with respect to the side chain of any particular residue. As with the parent complex and the D121A and D121N analogs (deMel et al., 1992), good electron density is seen for residues 1–113 of RNase 1–118 and for residues 114–124 of the tetradecapeptides, but there is no electron density for the redundant residues (Fig. 5). The intramolecular main chain hydrogen-bonding pattern (Fig. 5) is very similar to that seen in RNase A (Wlodawer & Sjölin, 1983), in RNase 1–118:111–124 (Martin et al., 1987), and in the two Asp 121 analogs (deMel et al., 1992). When comparing the relative location of corresponding atoms in two structures, a change in bond distance was considered significant if it was greater than 0.30 Å. This arbitrary limit is more than twice the standard deviation of the differences between the main chain hydrogen bond distances of residues 2–110 in the parent and F120L (0.11 Å) and in the parent and F120Y (0.10 Å).

Residues 65–72

The loop formed by the disulfide bond connecting half-cystine residues 65 and 72 moves away from the active site of the enzyme in the leucine, but not in the tyrosine, analog (Fig. 6; Kinemage 4). The electron density observed for residues 65–72 in F120Y is shown in Figure 7. The value of the RMS difference for the positions of the C_{α} atoms of residues 66–69 is 0.64 Å for F120L. In RNase A, RNase 1–118:111–124, and F120Y, there is a direct hydrogen-bonding network linking the side chains of Asn 67, Gln 69, and Asn 71; these links presumably help to maintain the desired geometry in this region. The reorientation of the loop in F120L breaks up this network: the OE1 of Gln 69 and the ND2 of Asn 71 no longer hydrogen bond directly; instead, each now forms a hydrogen bond to the same water molecule (WAT 221). The side chain of Lys 66 moves significantly, disrupting its hydrogen-bonding pattern with Asp 121 (see the section on Lysine 66, below) (Fig. 8; Kinemage 4).

¹ The coordinate sets for RNase 1–118:111–124 (file 1SRN), D121N (file 3SRN), D121A (file 4SRN), F120L (file 1SSA), and F120Y (file 1SSB) are available from the Brookhaven Protein Data Bank (Bernstein et al., 1977).

Fig. 2. Difference distance matrices. The matrices were constructed by subtracting the C_{α} distance matrix for the refined RNase 1–118:111–124 structure from that for each of the analogs and contouring at ± 0.50 Å. The scales at the bottom and side of each panel represent residue numbers. **A:** F120Y. **B:** F120L.

In the tyrosine analog, no significant overall movement of the 65–72 loop occurs; the RMS difference for the C_{α} atoms of residues 66–69 is 0.24 Å. The hydrogen bonds linking OE1 of the Gln 69 side chain to Asn 67 and Asn 71 remain, but the first bond is shortened (3.51 to 2.97 Å) and the second is lengthened (2.45 to 3.27 Å).

General features of the water structure

A total of 115 crystallographically bound water molecules have been identified in RNase 1–118:111–124 (deMel et al., 1992), using the criteria given in the Materials and methods. The num-

Fig. 3. RMS displacements compared to the parent structure. RMS displacements of main chain and side chain atoms: (A) F120L relative to those of RNase 1-118:111-124, and (B) F120Y relative to those of RNase 1-118:111-124, plotted as a function of residue number. The structures were overlapped using residues 2-110 and the program OVRLAP (Rossman & Argos, 1975).

ber of water molecules decreases to 109 in the F120L analog, with the majority of them occupying novel positions. A water position was considered novel if it differed from those seen in the parent complex by 0.30 Å or more. In F120Y, the number of bound waters decreases to 94. Of the waters comprising the first shell, that is, those waters within 3.5 Å of a nitrogen or oxygen of the protein, 26 are common between the parent structure and F120L, and 23 are common between the parent structure and F120Y. Only nine water molecules are common to all three structures. The changes in water structure are not confined to a region in the vicinity of residue 120, but are dappled over the surface of the protein.

Histidine 12

The positioning of His 12 has not been significantly disturbed by the replacement of Phe 120 with either leucine or tyrosine. As in RNase 1-118:111-124 and in the D121A and D121N analogs, the NE2 of His 12 in the leucine analog forms a short hydrogen bond to O4 of the sulfate at the active site. In F120Y, however, that bond has lengthened from 2.42 to 3.07 Å, and an additional long bond (3.45 Å) between NE2 and O3 of the sulfate has formed (Table 3).

Histidine 119

In RNase 1-118:111-124 and in both the Asp 121 analogs, the side chain of His 119 is found predominantly in a conformation first reported as a minor (20%) occupancy in RNase A crystal-

Fig. 4. Temperature factors. The average temperature factors for the four main chain atoms and for the side chain atoms of each residue are plotted as a function of residue number. A: F120Y. B: F120L.

lized from aqueous ethanol at pH 5.2-5.7 and denoted position B (Borkakoti et al., 1982, 1984). Subsequently, position B was seen to be the sole conformation for His 119 in crystals of a His 12 alkylated derivative of RNase A (Nachman et al., 1990) and in crystals of RNase S (Kim et al., 1992). Recently, a high-resolution NMR study of RNase A has revealed that position B also predominates in solution at pH 4.0 (Santoro et al., 1993). The chi 1 value for this position is between -52° and -60° , whereas that for the alternative conformation, position A, is between $+149^\circ$ and $+153^\circ$ (Kinemage 5). Position B is also the predominant conformation for His 119 in both the F120L and F120Y analogs. The positioning of the imidazole ring in the 120 analogs is not exactly coincident with that found in RNase 1-118:111-124, however, as the ring has shifted approximately 0.5 Å toward the sulfate in both cases. In neither case has the plane of the ring changed significantly, nor does the neighboring water structure indicate that the ring has flipped 180° , as was the case in the D121N analog (deMel et al., 1992).

Lysine 41

In both analogs, all the side chain atoms of Lys 41, an invariant residue thought to participate in the stabilization of the transition state (Hirs et al., 1965; Deavin et al., 1966; Roberts et al., 1969), are in good electron density. No significant change in the positioning of this side chain has occurred in either analog.

Glutamine 11

The hydrogen bond found in RNase 1-118:111-124 between NE2 of Gln 11 and the O3 of the sulfate is present in the F120Y analog but absent in the F120L analog.

Fig. 5. Backbone hydrogen-bonding scheme. Backbone hydrogen bonds with a donor-acceptor distance less than 3.50 Å and an angle greater than 110° are shown for RNase 1-118:111-124 (label = P), F120L (label = L), and F120Y (label = Y). Hydrogen bonds without any label are present in all three structures. The arrows go from donor to acceptor.

Aspartic acid 121

Of the three hydrogen bonds between Asp 121 and Lys 66 in RNase 1-118:111-124, namely NZ⁶⁶ to O¹²¹, NZ⁶⁶ to OD1¹²¹, and N⁶⁶ to OD2¹²¹, the last remains unchanged in the tyrosine

analog, but the NZ⁶⁶ to O¹²¹ bond has lengthened beyond our cutoff point of 3.50 Å (2.79 to 3.52 Å) and the NZ⁶⁶ to OD1¹²¹ bond has shortened (3.44 to 2.84 Å) (Table 3; Fig. 8). More drastic modifications have occurred in the leucine analog, where both the NZ⁶⁶ to O¹²¹ and NZ⁶⁶ to OD1¹²¹ bonds are gone and NZ⁶⁶ now hydrogen bonds to OD2¹²¹ (2.68 Å). In addition, a new interaction between the main chain N of Lys 66 and the OD1 atom of Asp 121 occurs (3.34 Å), owing to the movement of the entire 121 side chain toward the 65-72 residue loop (Table 3; Fig. 8).

Active site solvent molecules

Of the three water molecules found at the active site in RNase 1-118:111-124 (deMel et al., 1992), only WAT 138 is retained in the F120L and F120Y analogs; however, the nature and strength of the hydrogen bonds made by this remaining water are changed, generally in a way that is unique to each analog.

WAT 138 forms a short hydrogen bond to O4 of the sulfate in both the parent complex and F120L, but this bond is greatly lengthened in F120Y (Table 3). In both analogs, the strong hydrogen bond between WAT 138 and NE2 of Gln 11 remains, but it is lengthened; also, the bond between O2 of the sulfate and WAT 138 is unchanged, but in the F120L analog the bond with the O3 of the sulfate is no longer present (Table 3). The temperature factor for WAT 138 is substantially increased in both analogs (33 Å² for F120Y and 25 Å² for F120L versus 18 Å² for RNase 1-118:111-124), whereas this was not the case for the D121A (17 Å²) or the D121N (18 Å²) analog. A water molecule coincident with WAT 138 is also found in both refined structures of RNase A (Wlodawer & Sjölin, 1983; Borkakoti et al., 1984).

Active site sulfate anion

The sulfate anion found at the active site of RNase 1-118:111-124 is retained in both analogs, but its overall positioning is slightly different in each case (Kinemage 6). In F120Y, the sulfate anion has moved 0.24 Å relative to the parent and has rotated by 29° as measured by the ALIGN program (Satow et al.,

Fig. 6. Comparison of residues 65-72. The structure of the loop comprising residues 65-72 is shown in stereo for RNase 1-118:111-124 (solid line), F120Y (dotted line), and F120L (dashed line). The structures were overlapped using residues 2-110 and the program OVLAP (Rossman & Argos, 1975).

Fig. 7. Electron density for residues 65–72 in F120Y. Stereo view of the $2F_o - F_c$ density peaks for residues 65–72 in the tyrosine analog. The electron density is contoured at 2.0σ . For clarity, the electron density is given only to the extent of 2.0 Å around each residue.

1986). In F120L, the corresponding rotation is 0.53 Å and the overall rotation is also 29°, but around a different axis. Of the 11 hydrogen bonds formed in the parent complex by the four sulfate oxygens with water or with atoms of the protein, 7 remain in the tyrosine analog and only 5 in the leucine analog. The

five original hydrogen bonds remaining in F120L are also found in F120Y: the hydrogen bond between O4 of the sulfate and the main chain N atom of residue 120, the bond between O4 and NE2 of His 12, the bond between O4 and WAT 138, the bond between O2 of the sulfate and ND1 of His 119, and the bond

Fig. 8. Schematic diagram showing the hydrogen bonds between Lys 66 and residue 121 in RNase 1-118:111-124 and in the F120Y, F120L, D121N, and D121A analogs. The distances between NZ of Lys 66 and the sulfur of the sulfate anion at the active site are: RNase 1-118:111-124, 8.54 Å; D121A, 11.10 Å; D121N, 10.87 Å; F120L, 10.11 Å; F120Y, 7.79 Å.

Table 3. Hydrogen bond distances (Å) at the active site

Hydrogen bond	RNase 1-118:111-124	F120Y	F120L
His 12 ND1...Thr 45 O	2.68	2.60	2.78
His 12 NE2...Sul 125 O4	2.42	3.07	2.55
His 12 NE2...Sul 125 O3	—	3.45	—
His 12 NE2...WAT 138	—	—	3.30
His 119 ND1...Sul 125 O2	2.93	2.41	2.83
His 119 NE2...WAT 212	2.87	—	3.09
Lys 41 NZ...Gln 11 OE1	3.34	3.12	3.34
Lys 41 NZ...Asn 44 OD1	2.93	3.16	3.38
Lys 41 NZ...WAT 153	3.25	—	—
Gln 11 O...Asn 44 ND2	2.75	2.82	2.81
Gln 11 NE2...Sul 125 O3	2.81	3.11	—
Gln 11 OE1...WAT 141	—	3.24	—
Gln 11 OE1...WAT 132	3.37	—	—
Gln 11 OE1...WAT 183	—	—	—
Gln 11 NE2...WAT 138	2.66	3.17	2.92
Gln 11 NE2...WAT 228	2.83	—	—
WAT 138...Sul 125 O2	3.03	2.89	2.90
WAT 138...Sul 125 O3	3.39	3.50	—
WAT 138...Sul 125 O4	2.47	3.46	2.75
Sul 125 O3...WAT 214	—	3.40	—
Sul 125 O2...Phe 120 N	3.18	—	—
Sul 125 O1...Leu 120 N	—	—	3.48
Sul 125 O1...WAT 159	—	2.26	—
Sul 125 O1...WAT 214	—	2.51	—
Sul 125 O1...WAT 207	—	—	3.41
Sul 125 O1...WAT 213	—	—	2.73
Sul 125 O3...WAT 153	3.13	—	—
Sul 125 O3...WAT 228	2.47	—	—
Sul 125 O4...Phe 120 N	3.25	—	—
Sul 125 O4...Tyr 120 N	—	2.40	—
Sul 125 O4...Leu 120 N	—	—	2.98
Sul 125 O4...WAT 153	3.33	—	—
Lys 66 N...Asp 121 OD2	3.17	3.02	3.16
Lys 66 N...Asp 121 OD1	—	—	3.35
Lys 66 NZ...Asp 121 OD1	3.44	2.84	—
Lys 66 NZ...Asp 121 OD2	—	—	2.71
Lys 66 NZ...Asp 121 O	2.79	—	3.00
Lys 66 N...WAT 147	3.38	3.44	—
Lys 66 N...WAT 240	3.06	—	—
Lys 66 NZ...WAT 236	3.15	—	—

between O2 and WAT 138 (Table 3). The additional two hydrogen bonds retained in the tyrosine analog are between O3 and NE2 of Gln 11 and between O3 and WAT 138 (Table 3). The new hydrogen bond between O1 of the sulfate and the backbone N of Leu 120 and additional interactions between O1 of the sulfate and two water molecules complete the interactions seen in F120L to give a total of 8 hydrogen bonds (versus 11 in RNase 1-118:111-124). Four new hydrogen bonds in F120Y (including the interaction between O3 of the sulfate and NE2 of His 12 [3.45 Å]) result in a total of 11 hydrogen-bonding interactions for the sulfate anion in this case.

Leucine 120/tyrosine 120

In the leucine analog, the CB and CG atoms of the Leu 120 side chain are not coincident with the corresponding atoms of the Phe 120

side chain in the parent complex; they have moved 0.61 and 0.67 Å away from the sulfate, respectively. Also in this analog, the OG of Ser 123 has moved 2.34 Å toward the hole created by the loss of the phenyl ring at position 120. This movement might be expected to modify substrate binding (see the Discussion), but the value of K_M for cytidine 2',3'-cyclic phosphate has not changed (Table 1).

In the tyrosine analog, the oxygen of the phenolic hydroxyl of Tyr 120 accepts the proton of the Ser 123 hydroxyl group (2.92 Å), while the proton of the phenolic hydroxyl of Tyr 120 forms a strong hydrogen bond with WAT 178 (2.25 Å). A network is formed when this same water molecule also hydrogen bonds to the OG oxygen of Ser 123 (2.45 Å) (Fig. 9). Nevertheless, the temperature factor for WAT 178 is 42 Å². The plane of the phenyl ring of Tyr 120 remains unchanged from that assumed by Phe 120, but the ring has slid about 0.7 Å toward the active site.

Fig. 9. Observed hydrogen bonding interactions between Ser 123, Tyr 120, and WAT 178, as well as a postulated interaction of WAT 178 with the O4 of the uracil ring in uridine vanadate. The uridine vanadate molecule (dashed lines) was transferred into the F120Y coordinate system by overlapping the RNase A/uridine vanadate structure of Borah et al. (1985) (Protein Data Bank file 6RSA), using residues 2–110 of RNase 1–118 and the program OVRLAP (Rossman & Argos, 1975). Note that, in contrast to the Hodges–Merrifield model (Fig. 10), the actual structure involves a bridging water molecule between Ser 123 and position 4 of the nucleotide base.

Lysine 66

The orientation of Lys 66 in the F120Y analog is very similar to that seen in RNase 1–118:111–124 (Kinemage 4). However, detectable changes in the lengths of the two NZ hydrogen bonds made by this residue to Asp 121 have occurred in this analog (Table 3; Fig. 8). Owing to relative shifts in positioning, the NZ of Lys 66 is 0.75 Å closer to the active site (taken to be the sulfur of the sulfate anion at the active site) in F120Y than in the parent complex.

In the F120L structure, the Lys 66 side chain has moved out and away from the rest of the molecule, resulting in the rupture of the hydrogen bond between OD1 of Asp 121 and the NZ of Lys 66. Two new hydrogen bonds have formed between these two residues, namely NZ⁶⁶ to OD2¹²¹ (2.71 Å) and the main chain N⁶⁶ to OD1¹²¹ (3.34 Å) (Table 3; Fig. 8), but the NZ of Lys 66 is now 2.87 Å farther away from the sulfur of the sulfate anion at the active site than it is in the fully active parent complex.

Discussion

Many structural features of the leucine analog are reminiscent of those seen in two other catalytically defective analogs, D121N and D121A, generated by replacing Asp 121 with asparagine and alanine, respectively (deMel et al., 1992). In each case, a multiplicity of changes has occurred, most small but some not so small, and the majority at a considerable distance from the immediate area of the modification itself. These changes, which are greater in number and size than the structural variations previously noted between the parent complex and RNase A (Martin et al., 1987), include the movement of the loop containing residues 65–72 away from the active site (Fig. 6; Kinemage 4; deMel et al., 1992:Fig. 6), the rearrangement of hydrogen-bonding networks and solvent molecules at the active site, and a more or less generalized relocation of crystallographically bound solvent. Nevertheless, the major elements of secondary

structure remain undisturbed in all three analogs (Fig. 5; deMel et al., 1992:Fig. 5). The determination of the structure of F120Y, which is fully active or hyperactive, depending upon the substrate used (Table 4; Hodges & Merrifield, 1974), has provided a useful control concerning the degree to which the changes seen in the three catalytically defective analogs may be due to differences in measurements of the X-ray data or in the refinement procedures. A comparison of the difference distance matrices for the C_α positions (Nishikawa et al., 1972; Kundrot & Richards, 1987) of F120L (Fig. 2B) or of D121N and D121A (deMel et al., 1992:Fig. 9) with that of F120Y (Fig. 2A) indicates that the great majority of the changes seen in the three analogs are real. A comparable situation is seen in staphylococcal nuclease, where the 1,400-fold reduction in catalytic efficiency caused by replacing active site Glu 43 with an aspartate is accompanied by structural rearrangements throughout the molecule (Hibler et al., 1987; Wilde et al., 1988; Loll & Lattman, 1990).

The most striking structural disruption, common to all three catalytically defective analogs and not seen in the fully active

Table 4. Kinetic parameters and/or activities of F120Y and S123A at pH 6.0 with various substrates

Enzyme (substrate) ^a	K_M (mM)	k_{cat} (s ⁻¹)	Activity
RNase 1–118:111–124 (C>P)	1.1 ± 1.0 ^b	2.1 ± 0.8 ^b	—
F120Y (C>P)	3.1 ± 0.3 ^c	4.0 ± 0.4 ^c	97% ^d
F120Y (U>P)	—	—	176% ^d
S123A (C>P)	—	—	100% ^e
S123A (U>P)	—	—	42% ^e

^a C>P, cytidine 2',3'-cyclic phosphate; U>P, uridine 2',3'-cyclic phosphate.

^b deMel et al. (1992).

^c This study.

^d Hodges and Merrifield (1974).

^e Hodges and Merrifield (1975).

tyrosine analog, involves the interaction of the side chain of Lys 66, an invariant residue (Beintema et al., 1988), with Asp 121 (Fig. 8). In RNase A, a strong hydrogen bond (2.84 Å) is seen between NZ of Lys 66 and OD1 of Asp 121 (Wlodawer et al., 1982; Wlodawer & Sjölin, 1983), while in RNase 1-118:111-124 there is a bifurcated hydrogen bond linking NZ of Lys 66 to OD1 of Asp 121 (3.44 Å) and to the carbonyl oxygen of Asp 121 (2.79 Å) (Fig. 8; Martin et al., 1987). As these are the only noncovalent bonds linking the tip of loop 65-72 to the rest of the molecule, the displacement of elements of this loop upon mutating Asp 121 to asparagine or alanine was not surprising. We did not expect to see a similar effect upon replacing Phe 120 with leucine, however (Fig. 6). The occurrence of such a conformation in an analog where Asp 121 remains unchanged reveals that the normal positioning of loop 65-72 is not solely the result of hydrogen-bonding interactions between Asp 121 and Lys 66. In their structural study of Met 13 analogs of RNase S, Varadarajan and Richards (1992) also observed a displacement of loop 65-72 that was *away* from the rest of the molecule whether the replacement was larger (M13F) or smaller (M13G) than methionine.

The correlation between the displacement of loop 65-72 and the loss of catalytic efficiency in three different analogs clearly suggests that this disruption may be the structural basis for the activity loss, or at least a portion of it, in each case. The very similar reduction in catalytic efficiency in F120L and D121A to $273 \text{ s}^{-1} \text{ M}^{-1}$ and $250 \text{ s}^{-1} \text{ M}^{-1}$, respectively (Table 1), is consistent with this model. The catalytic efficiency of D121N is significantly less ($56 \text{ s}^{-1} \text{ M}^{-1}$), but there is an additional lesion unique to this analog: the imidazole ring of His 119 is flipped 180° in a majority of the molecules (deMel et al., 1992); this conformation would not be expected to be catalytically effective.

Earlier work by others indicated that a change in the positioning of loop 65-72 and, in particular, in the location of the side chain of Lys 66 might result in changes in enzymatic activity. In their study of sequence variation among mammalian pancreatic ribonucleases, Beintema et al. (1988) found several residues of this loop, including Lys 66, to be invariant across 40 species. Beintema (1989) has proposed that members of the ribonuclease superfamily must have a positive charge at position 66 or 122 in order to exhibit significant activity against the conventional RNase substrates. In RNase A and in our semisynthetic derivatives, which have alanine at position 122, this requirement is fulfilled by Lys 66. Proton NMR studies of RNase A reveal that the resonance position of the amide proton of Lys 66 responds to the titration of a group with a pK value of approximately 3, a situation ascribed to the existence of a hydrogen bond between the main chain nitrogen of this residue and the carboxylate group of Asp 121 (Rico et al., 1989). These authors noted that such a bond should be highly effective in anchoring together the two loops upon which these residues respectively reside.

Perhaps the epsilon amino group of the invariant Lys 66 assists in stabilizing the negatively charged pentacoordinate transition state (Roberts et al., 1969), a role it might share with Lys 41 (Deavin et al., 1966), and, in the three analogs in which Lys 66 has moved farther from the active site, this residue may be less well situated to perform this task. The shortest distance between NZ of Lys 66 in RNase A and an atom in the transition state analog, uridine vanadate (Lindquist et al., 1973), is 4.97 Å (to O5 of the ribose) (Borah et al., 1985; Protein Data Bank file 6RSA). Nevertheless, this distance should be near

enough to exert an appreciable electrostatic effect on the transition state. The value of k_{cat} for the hydrolysis of succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide by subtilisin BPN' was reduced successively from 57 s^{-1} to 45 s^{-1} and 30 s^{-1} when Asp 99 was replaced by serine or lysine (Russell & Fersht, 1987). The authors attributed the effect on k_{cat} of changes in this residue, which is 13 Å from the active site (His 64), to electrostatic destabilization of the transition state. Such a role for Lys 66 is supported by the observation that the NZ of this residue is 0.75 Å *nearer* the active site in F120Y, an analog exhibiting a twofold increase in the turnover of cytidine 2',3'-cyclic phosphate (Table 1).

When His 119 is in position A, one of the carboxyl oxygens of Asp 121 is within hydrogen-bonding distance of nitrogen NE2 (2.74 Å) (Wlodawer & Sjölin, 1981; Wlodawer et al., 1982; Protein Data Bank file 5RSA). In this conformation, Asp 121 also forms two good hydrogen bonds with Lys 66 (OD1 to NZ, 2.84 Å; OD2 to N66, 2.90 Å). If this hydrogen-bonding network has catalytic significance and if it has been modified in F120L, such a change might be the structural basis for the kinetic effects observed in this analog. All the structures reported here have His 119 predominantly in position B, however, so they are not germane to an assessment of this possibility.

The hydrogen-bonding network involving the phenolic hydroxyl of Tyr 120, the hydroxyl of Ser 123, and WAT 178 that is found in the F120Y analog seems to explain the kinetic properties previously observed for both F120Y (Hodges & Merrifield, 1974) and S123A (Hodges & Merrifield, 1975). The activity of F120Y toward uridine 2',3'-cyclic phosphate was enhanced (176% active; Table 4), whereas the activity of S123A toward this substrate was halved (Table 4). These authors had proposed the possible existence of a hydrogen bond between Ser 123 in RNase and elements of the pyrimidine ring in both uridine- and cytidine-containing substrates (cf. Fig. 10). Ser 123 was postulated to be a hydrogen bond donor with uridine-containing substrates and a hydrogen bond acceptor with cytidine-containing substrates. The lowered activity of S123A toward uridine 2',3'-cyclic phosphate was consistent with this hypothesis. Subsequently, in the crystal structure of the complex between RNase A and the transition-state analog, uridine vanadate, a hydrogen-bonding interaction was seen between the O4 of the uridine ring and the OG oxygen of Ser 123, but it was mediated by a water molecule (Borah et al., 1985; Protein Data Bank file 6RSA; cf. Fig. 9). From the structure of F120Y, we can now hypothesize that by accepting the hydrogen of the phenolic hydroxyl of Tyr 120, this intervening water molecule (our WAT 178) becomes a better hydrogen donor to the oxygen of the carbonyl group at position 4 of the uracil ring, with a consequent increase in catalytic efficiency.

Hodges and Merrifield (1975) found that the postulated role for the hydroxyl of Ser 123 in binding cytidine-containing substrates (Fig. 10) was not supported by the kinetics of S123A, however, as full activity was observed toward cytidine 2',3'-cyclic phosphate (Table 4). The finding that F120Y exhibited full activity toward this substrate (Table 4) did not seem germane to the hydrogen-bonding hypothesis at the time. We have now found, however, that both K_M and k_{cat} for cytidine 2',3'-cyclic phosphate are, in fact, significantly changed when tyrosine replaces Phe 120 (Table 1). (The changes can be countervailing in their effect on activity measurements; our values predict 85% activity under the assay conditions used by Hodges and Merrifield [1974], who observed 97% activity.) Although S123A ap-

Fig. 10. Hypothetical hydrogen-bonding interaction between Ser 123 and the pyrimidine ring of uracil- or cytosine-containing substrates as proposed by Hodges and Merrifield (1975:Fig. 1). In the Hodges-Merrifield model, Ser 123 interacts directly with the base, requiring that it function as a donor with uracil and as an acceptor with cytosine. The 4.87-Å distance between Ser 123 OG and the uracil O4 in the RNase A/uridine vanadate structure (Borah et al., 1985; Protein Data Bank file 6RSA) reveals that a direct hydrogen bond is not possible. In our model (see Fig. 9), an intervening water molecule functions as a donor or acceptor depending upon which base is presented, while Ser 123 always hydrogen bonds to this water as an acceptor whether the base is uracil or cytosine.

pears to be fully active toward cytidine 2',3'-cyclic phosphate, countervailing changes in kinetic parameters comparable to those seen in the tyrosine analog may also have occurred here. It seems that a hydrogen-bonding interaction of the type postulated to occur between the amino group of the cytosine base and the oxygen of Ser 123 (Hodges & Merrifield, 1975), but involving a bridging water molecule, may exist, in fact, and may be kinetically significant. When cytidine 2',3'-cyclic phosphate is the substrate, this water would function as the *acceptor* in a hydrogen bond with the amino group at position 4 of the cytosine ring (Fig. 10). In this case, its additional interaction with Tyr 120, also as a hydrogen bond acceptor, is detrimental to substrate binding.

Materials and methods

Materials

RNase A (Type XII-A) used in the preparation of RNase 1-118 was purchased from Sigma, as were carboxypeptidase A (Type I-DFP), its substrate, hippuryl-L-phenylalanine, pepsin (P-6887), and cytidine 2',3'-cyclic phosphate, sodium salt (C9630).

Preparation of RNase 1-118

RNase 1-118 was prepared according to previously published procedures (Doscher et al., 1983b), except that the gel-filtered product was further purified by isocratic ion-exchange chromatography at 5 °C on SP-Sephadex G-25, 40-120 µm (0.13 M Na phosphate, pH 6.65). Stock solutions of RNase 1-118 were characterized by amino acid analysis of acid hydrolysates of aliquot samples and by the determination of specific activity against cytidine 2',3'-cyclic phosphate, both in the absence and in the presence of a saturating level of RNase 111-124. Activity against this substrate was determined spectrophotometrically as described below.

Synthesis of RNase 111-124(F120L) and RNase 111-124(F120Y)

The tetradecapeptide analogs were synthesized by the Wayne State University Macromolecular Core Facility and purified by methods previously described (Sasaki et al., 1979; Doscher et al., 1983b). Stock solutions of the peptides were characterized and standardized by amino acid analysis of acid hydrolysates of aliquot samples.

Kinetic characterization of F120Y

The values of K_M and k_{cat} for F120Y and cytidine 2',3'-cyclic phosphate were determined at pH 6.00 and 25 °C using a spectrophotometric assay based closely on that of Murdock et al. (1966), which utilizes the increase in absorbance in the vicinity of 286 nm that occurs upon conversion to the product, 3'-cytidylate (Sasaki et al., 1985). The integrity of substrate solutions was checked by measurement at pH 7.00 of the ratio of the absorbance at 265 nm to that at 286 nm; a value lower than 2.94 indicated that hydrolysis to 3'-cytidylate had occurred (Crook et al., 1960). To ensure that measured velocities reflected complete conversion of RNase 1-118 to its complex with RNase 111-124(F120Y), velocity determinations were made in quadruplicate at four progressively higher mole ratios of RNase 111-124(F120Y) to RNase 1-118. The maximal velocity at saturating levels of peptide was then determined by extrapolation to the *y*-axis of a double reciprocal plot of velocity versus mole ratio.

Crystallization

Crystals of RNase 1-118:111-124 and the two analogs were all grown from ammonium sulfate/cesium chloride salt solutions buffered at pH 5.2 as described earlier (Sasaki et al., 1979; Doscher et al., 1983a). CsCl was removed by transferring the crystals into a stabilizing buffer of 80% saturated ammonium sulfate, 0.1 M ammonium acetate, pH 5.2. Individual crystals were soaked between 2 and 4 h, with several changes of solu-

tion, before being mounted in a glass capillary in the usual way. The crystals deteriorated noticeably if stored in the absence of CsCl longer than a week.

Data collection

Area detector data were collected for the tyrosine and leucine analogs on a Siemens system employing a three-axis camera, a PCS-driven controller, and the XENGEN software package (Howard et al., 1987) supported on a VAX 3600 minicomputer, using procedures identical to those used for D121A and D121N (deMel et al., 1992).

Refinement

Refinement with ROTLSQ, X-PLOR, PROLSQ, and PROFFT was handled almost identically as with the D121N and D121A analogs (deMel et al., 1992). A solvent molecule was added and retained if the electron density peak (1) appeared in $F_o - F_c$ maps at greater than 3.5σ , (2) appeared in $2F_o - F_c$ maps at greater than 0.7σ , and (3) its position was within hydrogen-bonding distance of an appropriate atom.

Hydrogen bonds

In evaluating hydrogen bonds, a bond length less than $3.50 \text{ \AA}$ and bond angle of greater than 110° were required for inclusion.

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