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The occupancy of two distinct conformations by active-site histidine-119 in crystals of ribonuclease is modulated by pH

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Abstract

Structures of a semisynthetic RNase have been obtained to a resolution of 2.0 Å at pH values of 5.2, 6.5, 7.5, and 8.8, respectively. The principle structural transformation occurring over this pH range is the conversion of the side chain of active site residue His-119 from one conformation ($\chi_1 = -43°$ to $-57°$) at low pH to another ($\chi_1 = +159°$ to $+168°$) at higher pH values. On the basis of this observation, a model is proposed that reconciles the disparate pK values for His-119 in the enzyme-substrate complex that have been deduced from kinetic studies and from proton NMR measurements in the presence of pseudosubstrates.

Key words: Mobile histidine; Modulation of conformation by pH; Protein semisynthesis; RNase mechanism of action; Semisynthetic RNase; X-ray structure

1. Introduction

Active site residue His-119 exhibits a conformational mobility that is unique among the amino acid residues in bovine pancreatic ribonuclease A (RNase A). By rotation about its C_α–C_β bond, the imidazole side chain can occupy two distinct conformations, denoted position A ($\chi_1 = +149°$ to $+168°$) and position B ($\chi_1 = -43°$ to $-60°$) [1–5]. The relative occupancy of the two conformations in the crystal appears to be modulated by the crystallization solvent, with position A being favored in aqueous organic solvents ([1,2], V.S.J. de Mel, unpublished) and position B being favored in salt solutions [3–5]. Baudet-Nessler et al. [6] found His-119 to be in position A in a fluorescent His-12-alkylated derivative of RNase A crystallized from salt solutions, but, in this case, the imidazole ring of His-119 is stacked on the naphthyl moiety of the His-12 substituent. In addition, Harris et al. [7] have reported that a non-bonded potential energy map for His-119 reveals two discrete side chain positions possessing energy minima; these positions correspond to the crystallographically observed A and B positions and are linked by a low energy pathway. Recently, a high resolution 2D NMR study of RNase A has revealed that position B also predominates in solution at pH 4.0 [8]. The equilibrium between the two conformations in solution appears to be a function of pH, with position B being favored at low pH values and position A being favored at high pH values [9].

Given the central role ascribed to His-119 in the catalytic mechanism of RNase [10–13], the possibility that its dual positioning has mechanistic significance is an attractive idea. In their studies of the active site dynamics of RNase A, Brünger et al. [14] noted that His-119 underwent dihedral-angle transitions in some of the simulations and suggested that this freedom of movement might be important for the catalytic mechanism. Modelling studies [15] have revealed, however, that His-119 is positioned equally well to participate in both the transphosphorylation and hydrolytic reactions catalyzed by the enzyme whether it is in position A or position B. As yet, no experimental verification or disproof of a linkage between the dual conformations of His-119 and the mechanism of action of the enzyme has been forthcoming.

A fruitful system for the study of RNase has been the fully active semisynthetic noncovalent complex formed between RNase 1–118 and a synthetic 14-residue peptide containing the C-terminus of the molecule, that is, residues 111–124 [16,17] (Fig. 1). The redundant residues in RNase 111–124 are needed to achieve good binding and full activity. The six C-terminal residues are unique to the peptide, however, allowing study of the possible roles played by these residues in generating the catalytic efficiency and substrate specificity of the enzyme through the chemical synthesis of structural variants. Semisynthetic complexes between RNase 1–118 and a large num-

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Abbreviations: RNase A, bovine pancreatic ribonuclease A; RNase 1–118, polypeptide consisting of residues 1 through 118 of RNase A; RNase 111–124, tetradecapeptide consisting of residues 111–124 of RNase A; RNase 111–124[Tyr-120], tetradecapeptide in which Phe-120 is replaced by Tyr; RNase 1–118:111–124, noncovalent complex of RNase 1–118 and RNase 111–124; (F120Y), noncovalent complex of RNase 1–118 and RNase 111–124[Tyr-120] in which Phe-120 is replaced by Tyr; UpA, uridylyl-3′,5′-adenosine; UpcA, UpA in which the 5′ oxygen of the ribose moiety of the adenosine is replaced by a methylene group.

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ber of structural analogs of RNase 111–124 have been prepared and characterized kinetically [17–24] and by proton NMR measurements [25–27]. In addition, high-resolution crystal structures have been determined for several catalytically modified analogs [15,28] as well as for the fully active parent structure [3].

We report here on the positioning of His-119 in four 2.0-Å structures of F120Y obtained at pH values of 5.2, 6.5, 7.5, and 8.8, respectively. F120Y is an analog in which the phenylalanine present at position 120 in the parent structure has been replaced by a tyrosine, resulting in an enzyme that is fully active or hyperactive, depending upon the substrate [19]. Crystals of this analog were used for the pH study because they proved to be more robust than crystals of the parent complex toward changes in the acidity of the mother liquor. We have found that the positioning of His-119 in the crystal is modulated by the pH of the mother liquor, with a predominant occupancy of position B converting to a predominant occupancy of position A over the pH range of 5.2 to 8.8.

2. Experimental

2.1. Preparation of RNase 1–118 and RNase 111–124[Tyr-120]

RNase 1–118 was prepared from RNase A (Type II-A, Sigma) by successive peptic and carboxypeptidase A digestions of the protein [16], and characterized as previously described [28,29]. RNase 111–124[Tyr-120] was synthesized by the Wayne State University Macromolecular Core Facility and purified as previously described for other peptides [29,30]. Stock solutions of the peptide were characterized and standardized by amino acid analysis of acid hydrolysates of aliquot samples.

2.2. Crystallization

Crystals of F120Y were grown from ammonium sulfate/cesium chloride salt solutions buffered at pH 5.2 as described earlier [25,29,31]. For the determination of the structure at pH 5.2, CsCl was removed by transferring individual crystals into a stabilizing buffer of 80% saturated ammonium sulfate, 0.1 M ammonium acetate, pH 5.2. The crystal was then soaked between 2 and 4 h, with several changes of solution, before being mounted in a glass capillary in the usual way. For the determination of the structures at pH values of 6.5, 7.5, and 8.8, crystals were transferred to 80% saturated ammonium sulfate that had been adjusted to the appropriate pH value. When crystals of the parent phenylalanine-120-containing enzyme were shifted from 80% ammonium sulfate, pH 5.2, to 80% ammonium sulfate, pH 7.5, they rapidly deteriorated. Consequently, the pH study was carried out with the fully active Tyr-120 analog, which seemed better able to stand the shock of the pH change. All the crystals deteriorated noticeably if stored in the absence of CsCl longer than a week.

2.3. Data collection

Area detector data were collected for the structures at pH values of 7.5 and 8.8 on a Siemens system employing a three-axis camera, a PCS-driven controller, and the XENGEN software package [32] supported on a VAX 3600 minicomputer, using procedures identical to those used for the determination of the structure of F120Y at pH 5.2 [28]. For the structure at pH 6.5, area detector data were collected at room temperature on the same Siemens system, but employing a four circle goniometer.

2.4. Refinement

The crystals belong to the same space group as the parent molecule [3], and, therefore, the refined coordinates for this molecule (Protein Data Bank [33], entry 1SRN) were used as the initial model. Rigid body refinement was followed by simulated annealing performed with the X-PLOR program [34]. Further refinement was carried out using the program PROLSQ [35] while checking the stereochemistry graphically using the program TOM/FRODO [36,37].

Water molecules were added and retained if the electron density peak, (1) appeared in F_o-F_c maps at greater than 3.2 σ, (2) appeared in 2 F_o-F_c maps at greater than 0.7 σ and (3) the position was within hydrogen bonding distance of an appropriate atom.

In the final stage of the refinement, a few cycles of PROFFT [38,39] and B-refinement in X-PLOR were used.

To determine the relative occupancy by His-119 of positions A and B at each pH value, independent refinement of the two structures with the side chain exclusively in position A or in position B was first carried out. In each case, His-119 side chain atoms were assigned full occupancy and the model subjected to a few cycles of PROLSQ refinement. 2 F_o-F_c maps were computed to check the electron density for the imidazole rings and to verify the refined A and B positions. Then the side chain atoms of His-12, His-105 and His-119 were removed, substituted by alanine, and a few cycles of PROLSQ refinement were carried out before computing F_o-F_c maps.

Integration of the difference electron density was performed by summing density that was greater than 2σ above background at all grid points within 1.9 Å of the CD2, NE2, and CE1 atoms of the imidazole ring. The 1.9-Å cutoff and the three ring atoms were chosen to exclude from the calculation any density common to the two conformations as well as any density from the C_β carbon or beyond. The relative occupancy of the alternate positions of His-119 was determined by calculating the ratio of the integrated densitites for the two positions.

3. Results and discussion

As the pH of the 80% saturated $(NH_4)_2SO_4$ surrounding the crystals of F120Y is progressively raised from

Table 1
Data collection and refinement

Statistics[a]	F120Y at pH 5.2	F120Y at pH 6.5	F120Y at pH 7.5	F120Y at pH 8.8
Unit cell (Å)	$a = b = 68.15$	$a = b = 67.77$	$a = b = 67.81$	$a = b = 67.13$
	$c = 65.15$	$c = 65.03$	$c = 65.01$	$c = 64.82$
	$\gamma = 120$	$\gamma = 120$	$\gamma = 120$	$\gamma = 120$
Reflections used	11,776	11,722	10,406	9,497
Resolution	5.0–2.0	5.0–2.0	5.0–2.0	5.0–2.0
R after PROLSQ	0.193	0.182	0.178	0.186
R after X-PLOR B refinement	0.184	0.168	0.169	0.175
RMS bonds	0.025	0.021	0.020	0.020
RMS ω angle	1.9	1.6	1.6	1.5

[a]All structures were determined with crystals grown from 1.3 M ammonium sulfate, 3.0 M cesium chloride, pH 5.2. For structure determinations above pH 5.2, crystals were soaked at the appropriate pH value as described in section 2. The space group is $P3_221$.

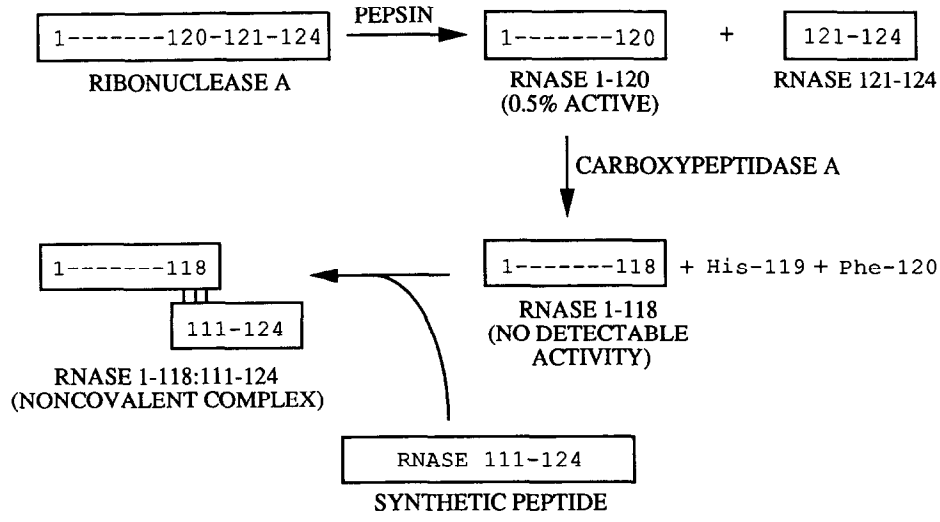

Fig. 1. Preparation and assembly of RNase 1–118:111–124. The schematic diagram shows the preparation of RNase 1–118 by proteolytic digestion and its assembly with a chemically synthesized 111–124 peptide into a noncovalent complex (which is fully active when no amino acid replacements have been made).

5.2 to 7.5 to 8.8, the unit cell dimensions diminish significantly (Table 1). Nonetheless, structures at the higher pH values refined readily when subjected to the rigid body refinement procedures described in section 2. The isoionic point of RNase A occurs at pH 9.6 [10], so the net positive charge on the molecule is declining over the pH range 5.2–8.8, a change that might be expected to allow greater compression of the structure. More muted unit cell expansions or contractions with changes in pH have been seen with other protein crystals [40–43].

As the pH increases from 5.2 to 6.5 and then to 7.5, small, but significant, increases occur in the occupancy of position A by the side chain of His-119 (Fig. 2, Table 2). These shifts are then followed by a more dramatic increase in the occupancy of position A as the pH is raised to 8.8 (Table 2). An additional structural change accompanying the rise in pH from 7.5 to 8.8 is the disappearance of the sulfate dianion from the active site. This loss of the sulfate ion may contribute to the greater degree of conformational shift seen upon going from pH 7.5 to pH 8.8. In contrast, no consistent change in occupancy with an increase in pH is seen with the uniquely positioned side chains of active site residue His-12 or of His-105, considered to be a 'normal' histidine residue [10,11] (Table 2).

Table 2
Variation of the occupancy of His-119 side chain conformers as a function of pH; comparison with two uniquely positioned histidine residues, His-12 and His-105[a]

pH	His-105	His-12	His-119 in position A	His-119 in position B	Position A/ Position B
5.2	9,864[b]	11,547	3,630 (0.42)[c]	5,021 (0.58)	0.7
6.5	11,215	12,658	3,271 (0.50)	3,233 (0.50)	1.0
7.5	8,445	11,130	3,197 (0.63)	1,866 (0.37)	1.7
8.8	9,631	12,819	8,529 (0.76)	2,681 (0.24)	3.2

[a] Calculated as described under Section 2.
[b] Total electron density above 2σ/atom.
[c] Fraction of side chain in this position.

The total electron density found for the side chain of His-119, that is, the sum of the electron density seen for positions A and B, is comparable at both pH 5.2 and 8.8 to that found for His-12 and His-105 (Table 2). At the intermediate pH values of 6.5 and 7.5, however, where k_{cat} values are maximal [44], the total electron density for His-119 is much smaller. No third conformation is evident at these pH values. Such a decline could indicate a greater side chain mobility at these pH values than at pH 5.2 or 8.8. The temperature factors for the side chain atoms are not increased in this pH range, however.

As judged from the difference distance matrix [45,46] for the C_α atoms of the pH 5.2 and pH 8.8 structures, no backbone structural changes such as the movement of the loop containing residues 65–72, which is seen in several catalytically modified analogs [15,28], are evident over the pH range of 5.2 to 8.8.

Analysis of the structure of RNase A by 2D NMR techniques has revealed the existence in solution also of the two conformations corresponding to positions A and B for the side chain of His-119 [8]. Moreover, the occupancy of the two conformations is modulated by the pH of the solution in this case as well, with the occupancy of position A being favored at higher pH values [9].

We propose that the equilibrium between the two conformations observed for the side chain of His-119, having quite similar energy levels, can be modulated by a moderate change in conditions, such as a variation in the local electrostatic potential. At pH 5.2, both the positive charge on the side chain of His-119 itself and the high net positive charge on the molecule as a whole will favor

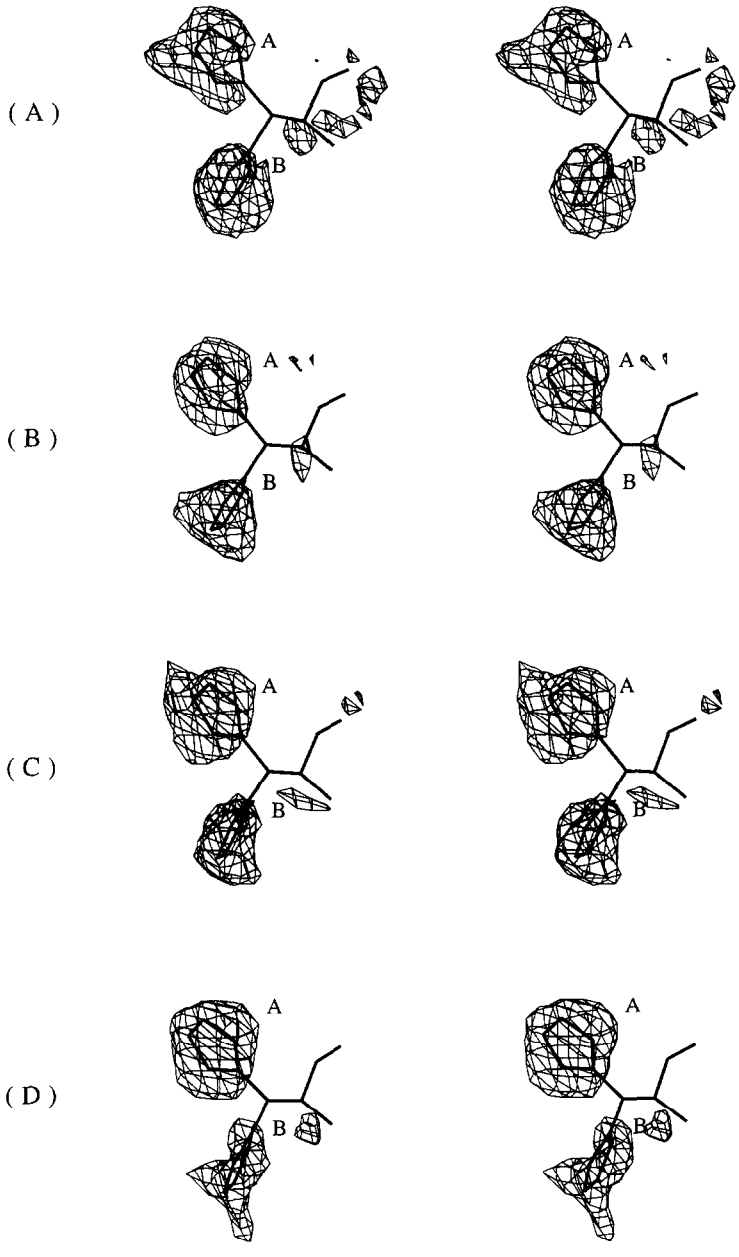

Fig. 2. Electron density for histidine-119 in F120Y at various pH values. The F_o-F_c density peaks for the imidazole ring of his-119 in F120Y contoured at 2ρ: panel A, pH 5.2; panel B, pH 6.5; panel C, pH 7.5; and panel D, pH 8.8. The conformations denoted position A and position B are shown schematically in each panel.

occupancy of position B. As the pH increases, both the positive charge on His-119 and the net positive charge on the molecule will decrease, allowing progressively greater occupancy of position A. Moreover, we propose that position A, but not position B, is the catalytically competent conformation for the hydrolysis of cyclic phosphate substrates. When the side chain of His-119 occupies position A, the molecule is an active enzyme, but when it occupies position B, the molecule is inactive.

Such a model can reconcile a major discrepancy between the values for the pK of the imidazole ring of His-119 assigned on the basis of kinetic measurements and those obtained from proton NMR studies in the presence of pseudosubstrates. On the basis of the pH dependence of the kinetic parameters for the hydrolysis of cytidine 2′,3′-cyclic phosphate, Findlay et al. proposed that the catalytically competent form of the enzyme contained one positively charged histidine and one neutral histidine at the active site [47]. To account for the occurrence of the maximal value of k_{cat} at pH 7.00, it was necessary to assign a pK value of 8.10 to the more basic dissociating group at the active site in the enzyme–substrate complex [44]. However, proton NMR measurements of RNase A in the presence of UpcA, a dinucle-

otide phosphonate analog of UpA that binds well to RNase, but cannot be hydrolyzed, owing to the replacement of the 5′-ribosyl oxygen of the adenosine by a methylene group, subsequently provided pK values of 6.1 and 5.8 for the side chains of His-12 and His-119, respectively [48]. It might be argued that replacement of the 5′-ribosyl oxygen with a carbon atom could perturb the pK value of His-119 significantly. Similar results were obtained, however, using the analog, 2′-deoxy-2′-fluorouridilyl (3′-5′) adenosine, which also binds well, and contains the 5′-ribosyl oxygen, but cannot be hydrolyzed owing to the replacement of the 2′-hydroxyl group with a fluorine atom [49]. With this analog bound, the corresponding pK values for His-12 and His-119 were found to be 6.60 and 6.71, higher than the values obtained with UpcA, but still far from the value of 8.10 proposed for His-119 by Herries et al. [44].

If, however, there is a progressively greater occupancy of the catalytically competent conformation for His-119 (position A) occurring over the pH range of 5.2 to 8.8, this event could partially compensate for the progressive decline in the required combination of one positively charged histidine and one neutral histidine, which is also occurring over much of this pH range. As these two factors have countervailing effects on the magnitude of k_{cat}, kinetic studies would measure the algebraic sum of the 'pK' for the pH-dependent conversion from position B to position A, an *activating* conversion, and the actual pK for the dissociation of the proton from the more basic imidazole ring, an *inactivating* conversion. As a result, the pK of His-119 in the enzyme–substrate complex would be assigned an erroneously high value.

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