NMR Study of the Positions of His-12 and His-119 in the Ribonuclease A-Uridine Vanadate Complex

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ABSTRACT The binding of uridine vanadate to ribonuclease A has been investigated by one- and two-dimensional ^{1}H NMR. The homonuclear Nuclear Overhauser and exchange spectroscopy spectrum of the uridine vanadate/RNase A complex exhibits cross peaks between both the C_5H and C_6H protons of uridine vanadate and the Hϵ1 proton of His-12 of ribonuclease A. These cross peaks suggest that the Hϵ1 proton of His-12 is in the vicinity of the uracil base of uridine vanadate, as observed in the crystallographic structure of the uridine vanadate/RNase A complex. However, no cross peaks are observed between the C_5H and C_6H protons of uridine vanadate and the Hϵ1 proton of His-119 of ribonuclease A, although they were predicted based upon the distances calculated from coordinates of the crystallographic structure of the complex. These results suggest that there is a significant difference between the positioning of the His-119 side chain in the solution and in the crystallographic structures.

INTRODUCTION

The structure and mechanism of ribonuclease A (RNase A), a small (13.7 kDa), highly stable enzyme that cleaves phosphodiester linkages in single-stranded ribonucleic acid chains, have been extensively studied (Richards and Wyckoff, 1971; Blackburn and Moore, 1982; Eftink and Biltonen, 1987). Since the publication of the first ^{1}H NMR spectrum for this protein (Saunders et al., 1957), many of the aromatic proton resonances have been assigned (Bradbury and Scheraga, 1966; Meadows et al., 1968; Bradbury and Teh, 1975; Markley, 1975a; Patel et al., 1975; Shindo et al., 1976; Lenstra et al., 1979) as well as a number of carbon resonances (Walters and Allerhand, 1980; Howarth and Lian, 1984). More recently, extensive assignments of backbone proton NMR resonances of RNase A (Robertson et al., 1989; Rico et al., 1989) have been made that confirm the assignments made by previous investigators. Moreover, a close similarity in the backbone structural elements is observed in the crystallographic and solution structures of the enzyme.

RNase A contains four histidine residues at positions 12, 48, 105, and 119 that are clearly visible in the aromatic region of its ^{1}H NMR spectrum. His-12 and His-119 lie within the active site of the enzyme (Richards and Wyckoff, 1971) and function as acid-base donors in the enzymatic mechanism (Findlay et al., 1962). The binding of substrates and inhibitors to the enzyme is often reflected in a change in the proton NMR resonances of these two residues (Meadows et al., 1969; Markley, 1975b; Markley and Ulrich, 1984). Recently, we reported the effects of replacing Asp-121 with Asn-121 on the titration behavior of the histidines in semisynthetic RNase A (Cederholm et al., 1991).

The two-step mechanism of RNase A begins with a transphosphorylation reaction that produces a cyclic 2′,3′-nucleotide intermediate, followed by a hydrolysis reaction that produces the 3′-monophosphate product. The transition state complex of both steps contains a pentacoordinate phosphorus. Uridine vanadate (U-V) is an analog (Lindquist et al., 1973) of the pentacoordinate transition state of RNase A. ^{1}H NMR studies (Borah et al., 1985) showed that U-V locks the enzyme in a specific conformation at physiological pH values. The proton NMR resonances of the Hϵ1 protons of His-12 and His-119 are each replaced by two new resonances that have been assigned as the protonated and unprotonated forms of the histidine resonances in a slow exchange process (Borah et al., 1985). The present studies suggest that the position of the His-119 side chain in this complex is significantly different in solution and in the crystal.

MATERIALS AND METHODS

Sample preparation

U-V was prepared using the method of Borah (Borah et al., 1985). A 60.8 mM sample of uridine was dissolved in 0.3 M NaCl and 0.5 mM 2,2-dimethyl-2-silapentane-5-sulphonate (DSS). Ammonium metavanadate was added to a concentration of 58.1 mM, and the sample was heated at 60°C for 15 min to form the U-V complex. Because U-V forms maximally at a uridine/vanadate ratio of about 1.2:1, the concentration of U-V is taken to be 50 mM.

An RNase A sample was prepared by dissolving 36.0 mg of the protein (Sigma R5500, Type XII-A) into 0.32 ml of 0.3 M NaCl and 0.5 mM DSS in D_2O at pH 5.5. The concentration of this sample determined spectrophotometrically was 8.21 mM using an extinction coefficient at 280 nm of 7.3 (Worthington Enzymes). The sample was heated at 60°C and pH 3.0 for 1 h to exchange the backbone amide protons. A 0.08-ml aliquot of the U-V sample prepared above was added to give a final composition of 6.57 mM RNase A and 10 mM U-V, concentrations of components that ensure enzyme saturation.

NMR measurements

All NMR measurements were carried out at 300 MHz on a Bruker AC-300 spectrometer at 30°C. The ^{1}H-NMR spectra were recorded with the following parameters: block size of 16K, a 60° pulse, a 4 s relaxation delay.

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Abbreviations used: NOESY, Nuclear Overhauser and exchange spectroscopy; RNase A, ribonuclease A; U-V, uridine vanadate; DSS, 2,2-dimethyl-2-silapentane-5-sulphonate.

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A typical spectrum was comprised of 800 scans. The two-dimensional NOESY spectrum of the U-V/RNase A complex was recorded at pH 5.5 using a phase-sensitive mode with presaturation of solvent (Bodenhausen et al., 1984). The spectrum was recorded in D_2O with a mixing time of 200 ms. Typically, 512 t_1 increments were collected, each with 2K data points over a spectral width of 3.6 kHz in the F1 direction and 1.8 kHz in the F2 direction. All chemical shift positions are relative to the principal resonance of 2,2-dimethyl-2-silapentane-5-sulphonate.

RESULTS AND DISCUSSION

The mechanism of RNase A that catalyzes the hydrolysis of single-stranded ribonucleotide phosphodiesters is believed to consist of a transphosphorylation by which the 3′,5′-phosphodiester is transesterified to a cyclic 2′,3′-phosphate and a hydrolysis by which the cyclic 2′,3′-phosphate is converted to a 3′-phosphate. In the transphosphorylation, His-12 acts as a base to remove a proton from the 2′ oxygen of the ribose, resulting in O2′ oxygen attacking the phosphorus. In the transition state, the phosphorus is pentacoordinate. His-119 acts as an acid to donate a proton to the O5′ end of the phosphodiester. In the second step of hydrolysis, His-119 acts as a base to accept a proton from water and then attacks the 2′,3′-cyclic phosphate, resulting in the formation of pentacoordinate phosphorus. His-12 then acts as an acid to donate a proton to the O2′ atom, thus forming the 3′-nucleotide (Richards and Wyckoff, 1971; Blackburn and Moore, 1982; Eftink and Biltonen, 1987).

The ^{1}H NMR resonances of the Hε1 protons of His-105, His-119, and His-12 of RNase A that have been previously characterized and assigned (Bradbury and Scheraga, 1966; Meadows et al., 1968; Bradbury and Teh, 1975; Markley, 1975; Patel et al., 1975; Shindo et al., 1976; Lenstra et al., 1979) are shown in the ^{1}H NMR spectrum in Fig. 1 *A* and exhibit chemical shifts of 8.68, 8.61, and 8.53 ppm, respectively, at pH 5.5. The resonance of the Hε1 proton of His-48 is only observed in the presence of acetate and propionate; in the presence of other salts, it is not observed due to slow exchange broadening near its pKa (Markley, 1975c). The ^{1}H NMR resonance of the Hδ2 proton of His-105 is also observed. However, in the presence of uridine vanadate, the ^{1}H NMR spectrum of RNase *A* is altered. The two Hε1 proton resonances of His-12 and His-119 in the spectrum of RNase A in Fig. 1 *A* are not observed in the spectrum of the U-V/RNase A complex in Fig. 1 *B*. Instead, four new resonances are observed at 8.84, 8.54, 7.86, and 7.45 ppm. The resonances at 8.84 and 7.86 ppm have been assigned as protonated and unprotonated forms of His-119, respectively, in a slow exchange process in the pH range 5–8, and the resonances at 8.54 and 7.45 ppm have been similarly assigned to the protonated and unprotonated forms of His-12, respectively (Borah et al., 1985). Thus, the ^{1}H NMR resonances of His-12 and His-119 change in intensity upon addition of uridine vanadate, suggesting that His-12 and His-119 are involved in complexing with uridine vanadate; in contrast, the ^{1}H NMR resonances of the Hε1 and Hδ2 protons of His-105 are not affected by the introduction of uridine vanadate, suggesting no comparable role in binding to uridine vanadate.

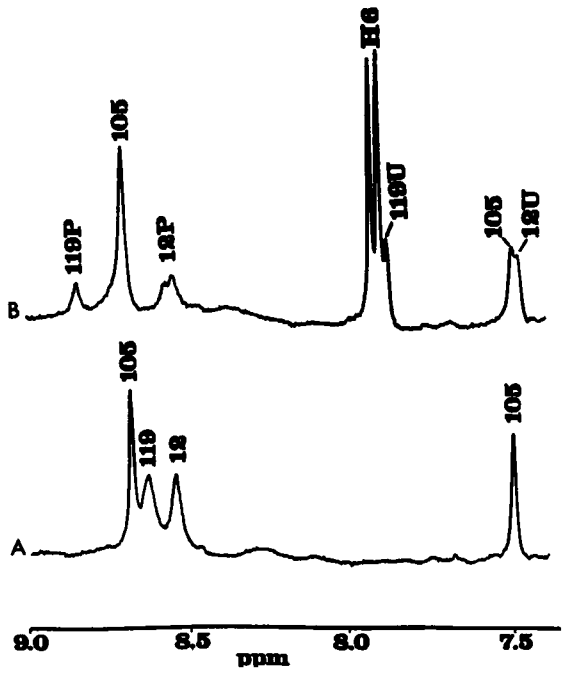

FIGURE 1 Proton NMR spectra at 300 MHz of (*A*) RNase A and (*B*) RNase A in the presence of uridine vanadate (U-V) at pH 5.5. The resonances of His-12 and His-119 in RNase A disappear when U-V is added to the protein sample. Four new resonances appear at 8.84, 8.54, 7.86, and 7.45 ppm that have been attributed to the protonated and unprotonated resonances of His-12 and His-119 (Borah et al., 1985). The resonances are labeled as follows: 119P, His-119 protonated; 119U, His-119 unprotonated; 12P, His-12 protonated; 12U, His-12 unprotonated; 105, His-105; H6, the proton on the C6 atom of uridine.

The contour plot of a homonuclear NOESY spectrum of the U-V/RNase A complex (Fig. 2) shows that cross peaks were observed between both the C_6H and C_5H resonances of U-V and the resonance assigned to the Hε1 proton of the protonated form of His-12, suggesting that uridine vandate is within approximately 5 Å of His-12. The reason for the absence of cross peaks corresponding to the interaction between the unprotonated form of His-12 and uridine vandate is not clear. Moreover, no cross peaks were observed between the C_6H and C_5H resonances of U-V and any of the resonances associated with His-119, indicating that uridine vanadate is not in the vicinity of His-119. Three additional cross peaks are observed. A cross peak is observed for the C_5H and C_6H protons of uridine vanadate, which are 2.42 Å apart, based upon the coordinates of the x-ray crystallographic structure of the U-V/RNase *A* complex (Borah et al., 1985). A second cross peak is observed for a phenylalanine resonance, possibly Phe-120, and the C_6H resonance of uridine vanadate, for which the distances range from 4.57 to 8.91 Å (Borah et al., 1985). A third cross peak is observed for the Hε1 and Hδ2 protons of His-105, which are 4.25 Å apart (Borah et al. 1985).

The x-ray crystallographic structure of RNase crystals grown in 60% ethanol, pH 5.2–5.7 shows well defined electron density for His-12 (Borkakoti et al., 1982); however, two possible orientations of His-119 were observed that differ by

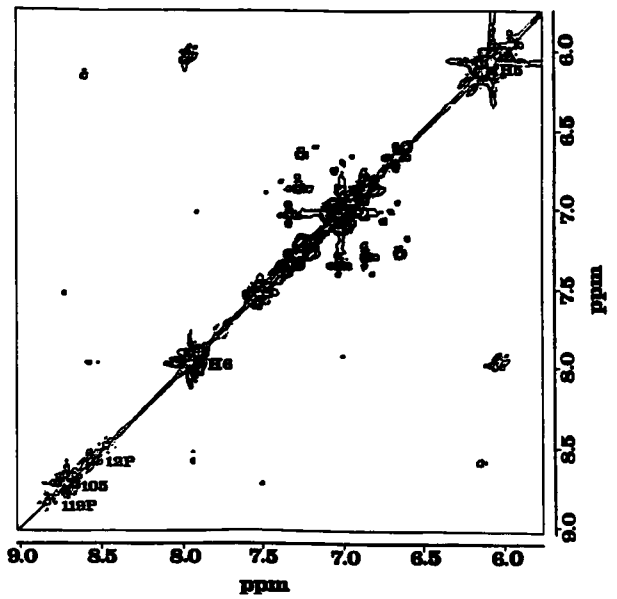

FIGURE 2 Contour plot of phase sensitive 2D-NOE spectrum of the aromatic region of U-V bound to RNase A at pH 5.5. RNase A and U-V concentrations are 6.57 and 10 mM, respectively, in 0.3 M NaCl and 0.5 mM DSS in D_2O. The spectrum was recorded at 30°C.

rotation about the C_α-C_β bond. In this particular structure, position A of His-119 exhibits a torsion angle χ_1 (N-C_α-C_β-C_γ) of 149°, a torsion angle χ_2 (C_α-C_β-C_γ-C_δ) of −101°, and an 80% occupancy, whereas Position B of His-119 exhibits a torsion angle χ_1 of −69°, a torsion angle χ_2 of −63°, and an occupancy of 20% (Fig. 3). In independent studies, the x-ray crystallographic structure of RNase *A* crystals grown in 43% t-butyl alcohol in the presence of phosphate, pH 5.3, (Wlodawer and Sjolin, 1983) indicate that His-119 is also primarily in position A, where the Nϵ2 of His-119 is

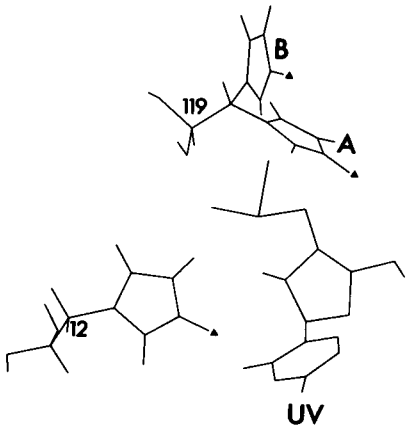

FIGURE 3 The active site residues His-12 and His-119 of RNase A in the presence of uridine vanadate (Protein data bank, reference 6RSA) (Bernstein et al., 1977; Wlodawer et al., 1983; Borah et al., 1985). In this crystallographic structure, His-119 occupies position A, which is labeled with an upper case letter A. His-119 was modeled in position B using torsional angles of χ_1 of −69°, and χ_2 of −63° (Borkakoti et al., 1982). The Cϵ1 protons of the His-12, of His-119 in the position A, and His-12 in position B, are labeled with a triangle (▲); uridine vanadate is labeled as UV.

hydrogen-bonded to the side-chain oxygen of Asp-121. Neutron diffraction studies (Borah et al., 1985; Wlodawer et al., 1983) and x-ray crystallographic studies (Alber et al., 1973) of a complex of RNase A and the transition state analog, uridine vandate, also place His-119 in position A. However, when native RNase A is complexed with the inhibitor O(3)--2′ CMP, His-119 is found primarily in position B, the second conformation that is achieved by rotation about the C_α-C_β bond (Palmer et al., 1984). As a consequence, His-119 does not hydrogen-bond to Asp-121 (Palmer et al., 1984), and the distance from the imidazole ring of His-119 to both the active site of the enzyme and the side chain of His-12 is several Angstroms greater than is seen in position A (Martin et al., 1987). In crystals of the fully active semisynthetic ribonuclease A (Martin et al., 1987) and of the Asn-121- and Ala-121-substituted semisynthetic ribonuclease analogs (de-Mel et al., 1992) that were grown in ammonium sulfate, pH 5.2, His-119 occupies primarily position B.

The combined neutron and 2 Å resolution x-ray diffraction analyses of the structure of the U-V/RNase A complex with *R* factors of 0.199 and 0.188, respectively, (Bernstein et al., 1977; Borah et al., 1985; Wlodawer et al. 1983) (Protein Data Bank, reference 6RSA) indicate that the distance between the Hϵ1 proton of His-12 and the C_6H proton of U-V is 5.48 Å and that the distance between the Hϵ1 proton of His-12 and the C_5H proton of U-V is 6.68 Å. Although these distances approach the upper limit of inter-proton distances normally observed by through-space correlation, the observation of cross peaks suggests that on a time average, these protons are close to each other. However, based upon the x-ray crystallographic coordinates of the U-V/RNase A complex, the distance between the Hϵ1 proton of His-119 and C_6H proton of U-V is 4.87 Å, and the distance between the Hϵ1 proton of His-119 and C_5H proton of U-V is 6.22 Å. The similarity of these distances to the distances between His-12 and U-V suggests that if two cross peaks are observed for C_6H proton of U-V and the Hϵ1 proton of His-12, and the C_5H proton of U-V and the Hϵ1 proton of His-12, then a cross peak should also be observed between the C_6H proton of U-V and Hϵ1 proton of His-119. The lack of such a cross peak suggests that a significant difference exists between the crystallographic and solution structures of the complex. When His-119 in the x-ray crystallographic structure of the U-V/RNase A complex is swung into position B with torsional angles, χ_1 of −69° and χ_2 of −63° (Borkakoti et al., 1982), the shortest distance between the Hϵ1 proton of His-119 and either proton of uridine vanadate is 9.56 Å, which would explain the absence of an observed cross peak for these resonances. Variations of χ_2 do not significantly reduce this value. Discrepancies in the x-ray crystallographic and solution structures of other proteins have previously been observed; however, they usually occur on surface residues, presumably due to protein-protein contacts found in the crystals (Bax, 1989; Wagner, 1990). The molecular packings of six crystal forms of pancreatic ribonuclease (Crosio et al., 1992), grown in t-butanol, ethanol, ammonium sulfate, and polyethylene glycol 4000, have been compared. In these six forms, active site His-119

is observed to occur in position A or position B and is highly solvent-accessible (Lee and Richards, 1971). The comparison of these six different molecular packings of RNase indicates that His-119 is not involved in any intermolecular contacts, thereby suggesting that the effects of crystal packing are not responsible for the differences in His-119 positions observed herein.

The two orientations of His-119 may be important to the two-step mechanism of RNase A, in which His-119 functions first as a general acid during the transphosphorylation step and subsequently as a general base during the hydrolysis step. Based upon relative proximity to the apical oxygen atom in models of RNase A complexed with the substrate analogue, UpcA, and with the transition state analog for the hydrolysis step, uridine vanadate, deMel and co-workers (deMel et al., 1992) have postulated that His-119 is more effective during transphosphorylation when it is in position B, and more effective in hydrolysis when in position A where a hydrogen bond to Asp-121 enhances its basicity. Although there is no NMR evidence to support the existence of the two separate orientations of His-119 of RNase A in solution, which may be due to interconversion between the two orientations occurring in a sufficiently rapid time scale, NMR data can provide evidence to support a certain orientation. A recent report (Rico et al., 1991) describes a similar discrepancy in the position of His-119 between the crystallographic and solution structure of RNase A complexed with phosphate. A strong NOE between one of the methyl resonances of Val-118 and the Hϵ1 proton of His-119 was observed, which is not predicted by the crystallographic structure (Borah et al., 1985) with His-119 primarily in position A; it was concluded that His-119 might be occupying an alternate position when phosphate is bound to the enzyme. We see a weak interaction between one of the methyl resonances of Val-118 and the Hϵ1 proton of His-119 of RNase A in the presence of uridine vandate, which is expected because the distances between these two protons range from 5.5 to 8.2 Å when His-119 is in position B, and range from 7.7 to 11.6 Å when His-119 is in position A using Protein Data Bank file 6RSA and the torsional angles listed above (Bernstein et al., 1977; Borah et al. 1985; Wlodawer et al. 1983). The strong NOE previously observed (Rico et al., 1991) cannot be explained in terms of His-119 simply being in position B. These observations suggest that the position of His-119 of RNase A is differentially affected by the presence of phosphate or uridine vanadate. Overall, the results suggest that the position of His-119 of RNase A in the presence of uridine vanadate observed crystallographically differs from that observed by NMR spectroscopy.

The observed differences in position A and B of His-119 of RNase A might be a function of differences in crystallographic and solution structures; however, they might be a result of differences in experimental conditions. The RNase A that exhibits His-119 primarily in the A position (Borkakoti et al., 1982; Wlodawer and Sjolin, 1983; Wlodawer et al., 1983) was crystallized from alcohols, such as 40–60% ethanol at pH 5.2–5.7, and 43% t-butyl alcohol at pH 5.3; in contrast, RNase crystallized from salt solutions such as ammonium sulfate at pH 5.2 (Martin et al., 1987; deMel et al., 1992) exhibits His-119 in the B position. Moreover, NOE spectra of the RNase A/phosphate complex in aqueous solution in 0.2 M NaCl at pH 4.0 show His-119 predominantly near Val-118, which abuts the B position (Santoro et al., 1993). Our results predict that a crystallographic structure of RNase A complexed with uridine vanadate in aqueous, ionic solution would have a significant fraction of the His-119 side chain in the B position.

It has been postulated that uridine vandate is a better analog of the transition state for the hydrolysis step than for the transphosphorylation. This implies that His-119 in the uridine vanadate complex should be closer to position A, which is not observed in our NMR experiments. Perhaps the greater ionic strength of the NMR sample weakens the interaction between His-119 and Asp-121 and allows the former to move away, possibly closer to the axial oxygen, which has a larger electrostatic potential than the equatorial ones (Krauss and Basch, 1992).

Molecular dynamics calculations (Brooks et al., 1986) would suggest that His-119 in the A position is important for hydrolysis because it is hydrogen-bonded to Asp-121, thereby making it a better base for abstracting the proton off the water that attacks the tetrahedral phosphonate in the cyclic nucleotide. *However*, the lack of this Asp-121 hydrogen-bonded to His-119 does not completely eliminate enzymatic activity. The replacement of Asp-121 by Ala results in a decrease of activity; the k_{cat}/K_M is *1.91* $mM^{-1}\ s^{-1}$ for semisynthetic wild-type RNase A and 0.25 $mM^{-1}\ s^{-1}$ for Ala-121 substituted semisynthetic RNase A (deMel et al., 1992). Moreover, the loss of hydrogen bonding capability between Asp-121 and methylated His-119 in semisynthetic RNase A results in neither a change in the pK_a of His-119 (Serdijn et al., 1984a, b) nor a total loss of enzymatic activity.

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