Structure of the Ribonuclease·Uridine−Vanadate Transition State Analogue Complex by Raman Difference Spectroscopy: Mechanistic Implications

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Abstract: Raman difference spectroscopy is used to assess changes in the internal bonding of the oxygens of a $\gg VO_2^-$ group when that group is incorporated into a complex also involving RNase A, uridine, and a water molecule (the RNase/UVO_2/H_2O complex). We find that the strengths of the nonbridging V···O bonds are decreased by 0.055 vu and their bond lengths are decreased 0.012 Å, based on the stretching frequency changes of nonbridging V···O bonds upon formation of the enzymic RNase/UVO_2/H_2O complex from the cyclic vanadate diester in solution. The bond lengths are 1.638 Å for the solution complex and 1.650 Å for the enzymic complex. The values found for the bond lengths are about 0.15 Å shorter than those found previously in crystallographic studies. Assuming the RNase/UVO_2/H_2O adduct is a reasonably good transition state analogue, our Raman results suggest that the RNase-catalyzed hydrolysis of uridine 2′,3′-cyclic phosphate proceeds via an S_N2-like process. The process also may involve a small associative charactor, since the summed bond strength of nonbridging P···O bonds is reduced by only 0.11−0.22 vu in the transition state compared with the ground state, which means the summed bond strength of the PO bonds of the entering and leaving groups is correspondingly increased.

Ribonuclease A, RNase, catalyzes hydrolysis of the RNA phosphodiester linkage in two steps, as shown in Scheme 1. In the first step, the phosphodiester bond is cleaved, resulting in a RNA strain with a free 5′-OH group (ROH in the second panel of Scheme 1), and a second RNA fragment which forms a 2′,3′-cyclic phopshodiester at the 3′ end. The cyclic intermediate is then hydrodrolyzed, converting the 2′,3′-cyclic diester into the 2′-phosphate monoester as depicted in the last panel of Scheme 1.

The cyclic vanadate diester anion complex with RNase A, where the phosphorus atom has been replaced by a vanadium atom in the molecular model of panel 3 of Scheme 1 (represented herein by RNase/UVO_2/H_2O), has been proposed as a transition state mimic of the pentacoordinate phosphorus expected in the transition state for hydrolysis of uridine 2′,3′-cyclic phosphodiester.[1,2] On one hand, X-ray crystallographic studies of the inhibitor complex are consistent with the pentacoordinated structure.[3] In other studies, the transition state binding paradigm was used (cf. ref 4). This paradigm requires that the affinity of an enzyme for a perfect transition state analogue exceed its affinity for the substrate by a factor equivalent to the increased catalytic rate in the enzymatic reaction relative to the corresponding nonenzymatic reaction. This latter factor has been estimated at 10^{10}-fold for RNase, based on the hydrolysis of cytidine 2′,3′-cyclic phosphate[5] or 10^9-fold based on the uridine derivative.[6] The equilibrium constant for forming the uridine−vanadate complex of RNase initially was thought to be only about 1000-fold larger than that of uridine 2′,3′-cyclic phosphate.[2] However, glycol−vanadate adducts, which also are considered as cyclic vanadate diesters, exist almost exclusively as dimers in aqueous solution. This seems likely with uridine as well.[7] In fact, the affinity of RNase for the *monomeric* pentacoordinate adduct relative to the cyclic phosphate probably is more than two orders of magnitude larger than originally estimated, i.e. at least 10^5-fold stronger than the related substrate because of extensive dimerization in solution (W. Ray, unpublished). Thus, the RNase/UVO_2/H_2O complex is a plausible transition state mimic based on both its crystal structure and the transition state binding paradigm.

Herschlag[8] has recently reviewed the chemical literature on the RNase reaction. He concludes that the most likely mechanism for the RNase-catalyzed hydrolysis of 2′,3′-cyclic phosphates involves general catalysis of an S_N2-like displacement, where the bond-breaking and bond-making processes resemble those that occur during the solution hydrolysis of phosphate

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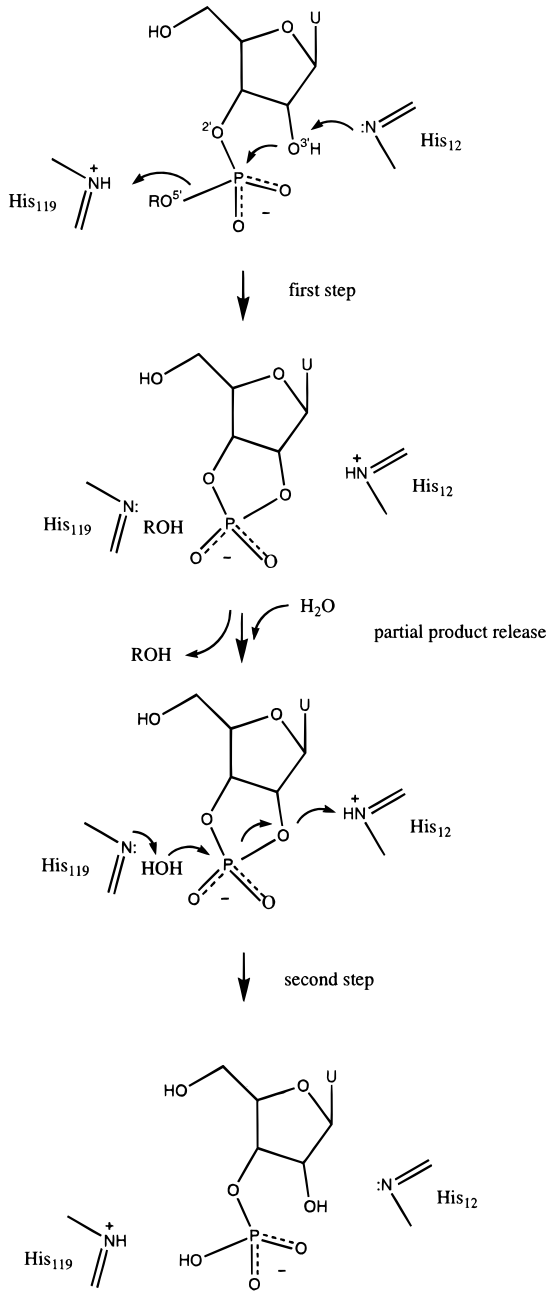
Scheme 1. The Two-Step Pathway for RNA Cleavage by Ribonucleases[a]



[a] The active site histidine residues act as proton donor−acceptors in both steps.

diesters with a good leaving group (cf. Hengge and Cleland[9]). A recent study of the heavy (^{18}O) atom kinetic isotope effects for the RNase A catalyzed reaction is in accord with this analysis.[10] Bond making and bond breaking in such processes are approximately synchronous, and the bond orders of the corresponding nonbridging P⋯O bonds are not affected much relative to the ground state.

However, the published X-ray structure of the RNase/UVO_2/H_2O complex is not consistent with the transition state of a S_N2-like reaction. The reported lengths of the nonbridging V⋯O bonds are substantially longer than those in a normal vanadate diester, which suggests that the strengths of the nonbridging P⋯O bonds in the transition state are substantially reduced relative to those in the ground state. This is important since the strength of the nonbridging P⋯O bonds would be reduced in the transition state in a stepwise associative process where a phosphorane-like adduct is formed, such as proposed by Breslow.[11]

Since the authors of the published X-ray structure of the RNase/UVO_2/H_2O complex indicated that they were unable to refine reliably the structure of vanadate moiety,[3] we have reexamined the RNase/UVO_2/H_2O complex by vibrational spectroscopy to assess the bond lengths and strengths of the nonbridging V⋯O bonds. Recent studies show that Raman spectroscopy is useful for estimating the lengths and strengths of nonbridging V⋯O bonds of vanadates in aqueous solution[12] and also in complexes formed with enzymes and vanadates.[13] Such estimates are based on empirical relationships relating frequency to bond strength and bond length. For some types of bonds, these relationships can provide bond lengths that are comparable in accuracy to small molecule crystallography, much better than that currently achievable in the crystallography of proteins (see Results).[12,14,15] Therefore, changes in bond lengths when a molecule binds with protein can be determined with high precision. Moreover, the results are from molecules or proteins in solution which, hence, do not suffer from possible distortions induced by crystallization.

Material and Methods

Uridine, 2′-deoxyuridine, and V_2O_5 were purchased from Aldrich and [^{18}O]water from CIL; all were used without further purification. NaH_2VO_4 (1 M) stock solution was prepared by stirring V_2O_5 with 2 equiv of aqueous NaOH overnight at room temperature. The uridine−vanadate complex was prepared by mixing uridine and NaH_2VO_4 solutions in water (or in 100 mM Tris, pH 7.5) to give final concentrations of 150 and 100 mM, respectively. RNase A, lyophilized and phosphate free, was purchased from Worthington and further purified by ion-exchange chromatography to remove fluorescent material. This was done by loading a 100 mg sample of RNase A onto a 1 × 25 cm A50 column equilibrated at pH 6.5 and 4 °C with 10 mM Tris buffer. The column was eluted with a linear NaC1 gradient, 0−400 mM, in 10 mM Tris, pH 6.5: flow rate, 0.5 mL/min. The major band in the elution profile (monitored at 280 nm) was collected and concentrated to about 10 mM in a centrifugal concentrator ($\epsilon_{278} = 9800$ M^{-1} cm^{-1}) and dialyzed overnight against 25 mM HEPES buffer at pH 7.5 and 4 °C. The RNase/UVO_2/H_2O complex was prepared by adding a concentrated stock solution of uridine (or deoxy uridine) and NaH_2VO to the enzyme solution: final concentrations of RNase, uridine (or deoxyuridine), and NaH_2VO_4 were 8−9, 10, and 6 mM, respectively. Labeling of the oxygens in the nonbridging V⋯O bonds of $(UVO_2)_2^{2-}$ or RNase/UVO_2/H_2O was achieved by preparing the above samples in ^{18}O water.

The difference Raman spectrometer has been described.[16] A 100 mW argon ion laser (514.5 nm) serves as the light source. A half-wave retarder was used to polarize the incident light either parallel or perpendicular to the entrance slit of the spectrometer. The symmetry of a vibrational mode was assessed in terms of the ratio of Raman intensities obtained with the excitation beam in the perpendicular and parallel configurations. In this optical arrangement, the intensity ratios

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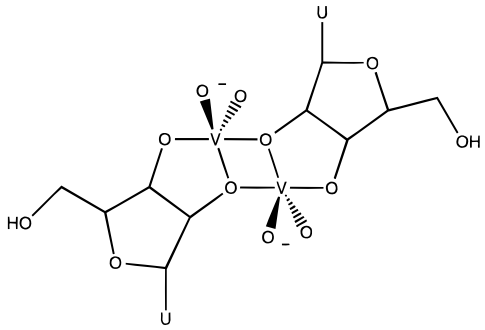
Chart 1. Structure of the $(UVO_2)_2^{2-}$ Complex



are 6/7 for an asymmetric mode and <6/7 for a symmetric mode for the optical configuration used here.[17] (This geometry, rather than the more common one that determines the "depolarization ratio", was employed to maximize signal throughput.) The polarization response of the spectrometer was calibrated by measurements of the toluene spectrum, where the symmetry of the various bands is known. The resolution of the spectrometer was set at 8 cm^{-1}, and band positions are accurate to ± 3 cm^{-1}. Fundamental frequencies were calculated as described in the final section of Results.

Results

Raman Spectra of the $\geqslant VO_2^-$ Group of the Dimeric Uridine/Vanadate Adduct. The structure of the $\geqslant VO_2^-$ groups in the uridine−vanadate dimer, $(UVO_2)_2^{2-}$, is shown in Chart 1 (cf. Angus-Dunne *et al.*[18] and Ray *et al.*[19]). Spectra a and b in Figure 1 are Raman spectra of the $\geqslant VO_2^-$ group of this dimer prepared in ^{16}O- and ^{18}O-water, respectively. In the latter case, only the two nonbridging oxygens in both pairs of $\geqslant VO_2^-$ groups exchange with solvent,[19] and thus only these are labeled. The 45 cm^{-1} decrease in frequency from 934 to 889 cm^{-1} upon ^{18}O labeling shows that the prominent bands in Figure 1 do indeed arise from vibrational modes of the $\geqslant VO_2^-$ groups. The inset below spectrum a in Figure 1 shows the Raman spectrum of the same sample obtained with perpendicularly polarized light. In such a spectrum, the intensity of symmetric modes is suppressed relative to asymmetric modes by known ratios (see Materials and Methods). Thus, the band at 912 cm^{-1} in Figure 1a, inset, which also appears as a shoulder on the lower frequency side of the main band in Figure 1a, can be assigned to the asymmetric nonbridging V⋯O stretching mode on the basis of its large intensity (incident light parallel/perpendicular) ratio of about 0.7. Similarly, the band at 934 cm^{-1} can be assigned to the symmetric nonbridging V⋯O stretching mode on the basis of its small intensity ratio of about 0.3.

Introducing ^{18}O into the nonbridging positions differentially alters both the frequency and Raman cross section of the symmetric and asymmetric stretching modes and, thus, aids in distinguishing these. For example, in tetravanadate, ^{18}O labeling reduces the intensity of the Raman band of the symmetric V⋯O stretching mode by about one-third, whereas the intensity of the asymmetric mode is little changed. In addition, as shown in Figure 1, the ^{18}O induced shift in the asymmetric stretching mode, about 30 cm^{-1}, is significantly less than that of the symmetric V⋯O stretching mode, about 45 cm^{-1}.

Raman Spectra of the Ribonuclease/Uridine−Vanadate Complex. Figure 2a shows the Raman difference spectrum of

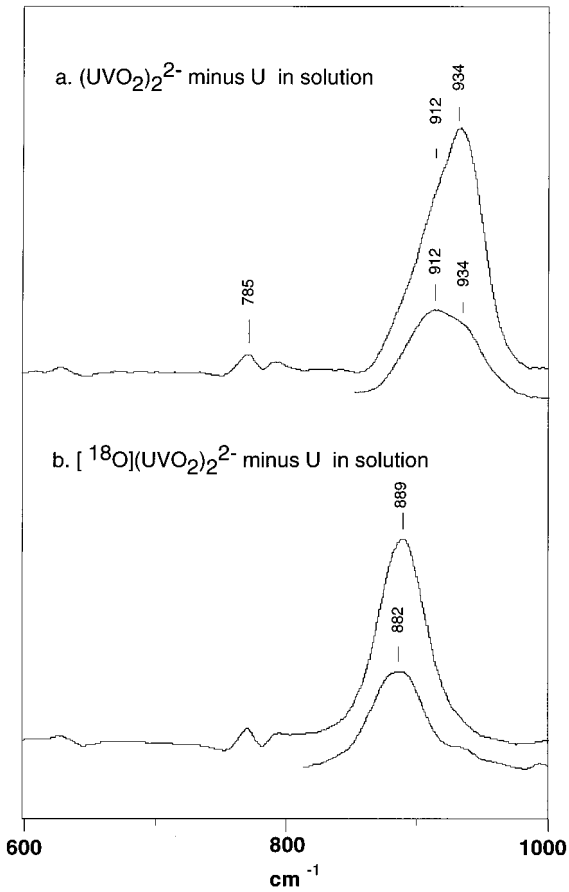


Figure 1. (a) Raman spectrum of the $\geqslant VO_2^-$ group of the uridine/vanadate dimer complex obtained with parallel polarization of the excitation beam (top spectrum) and spectrum obtained with a perpendicularly polarized beam (bottom). The sample contained uridine (150 mM) and NaH_2VO_4 (100 mM) in 100 mM Tris buffer, pH 7.5. Buffer and uridine peaks in the spectrum of an analogous sample, but without vanadate, were subtracted before plotting. (b) Same as in (a), except the sample was prepared in ^{18}O water.

a solution containing RNase, uridine, and vanadate minus that of a solution containing RNase and uridine. In the former solution, RNase was present in excess and uridine in large excess relative to vanadate. Essentially all of the vanadate in the former solution was present as the RNase/UVO_2/H_2O complex according to published binding constants;[6] in the RNase + uridine solution, about half of the RNase was present as its complex with uridine. Figure 2a was obtained with parallel polarized incident light while the graph just below Figure 2a is the analogous difference spectrum obtained with the same samples but using perpendicularly polarized light. This difference spectrum contains bands from the bound vanadate plus extraneous bands that arise from changes in vibrational modes of either protein or uridine bonds that are produced by forming the adduct. For example, the derivative feature at 785 cm^{-1} (Figure 2a) is produced by a uridine band that shifts to slightly higher frequencies in the adduct. When 2′-deoxyuridine is substituted for uridine, only Raman bands characteristic of inorganic vanadate and monoesters thereof are obtained.

The most intense peak in the Figure 2a difference spectrum, at 893 cm^{-1}, is about 7% as intense as the most prominent band in the spectrum of RNase. This band is assigned to the symmetric stretching mode of the V⋯O bonds in the $\geqslant VO_2^-$ group of the RNase/UVO_2/H_2O complex on the basis of four observations: the decreased intensity and frequency produced by ^{18}O labeling (Figure 2b); the shape and intensity of the band,

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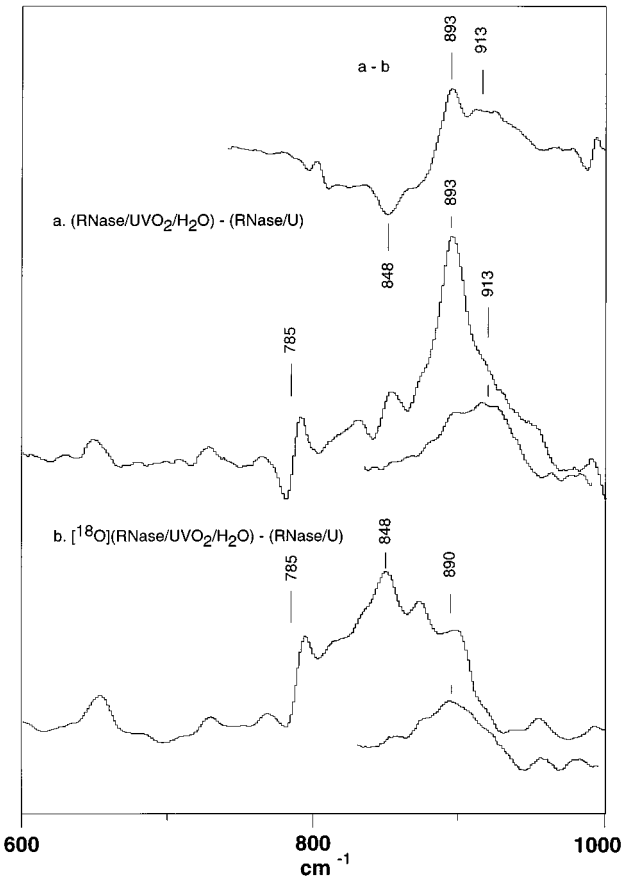

Figure 2. (a) Raman spectrum of the $\gtrdot VO_2^-$ group in RNase/UVO_2/H_2O obtained with parallel polarization of the excitation beam (top) and spectrum obtained with a perpendicularly polarized beam (bottom). The sample contained 8−9 mM RNase, 10 mM uridine, 6 mM NaH_2VO_4, and 25 mM hepes, at pH 7.5, and was maintained at 4° during data collection. The spectrum of an analogous sample without NaH_2VO_4 was subtracted before plotting. (b) Same as in (a), except that the sample was prepared in ^{18}O water. The insert above panel a is the difference spectrum formed by subtracting the parallel polarized ^{18}O spectrum from the ^{16}O spectrum.

which is analogous to the shape and intensity of the 934 cm^{-1} band of $(UVO_2)_2^{2-}$ in Figure 1a when the contribution of the 912 cm^{-1} shoulder is subtracted; the decrease in band intensity in a spectrum obtained with perpendicularly polarized light (inset below Figure 2a); and the absence of the band when 2′-deoxyuridine is used in place of uridine (data not shown). Although the 893 cm^{-1} band shifts toward the red on labeling with ^{18}O, Figure 2b, the extent of the shift cannot be specified unambiguously because there are several comparable protein bands in this region of the difference spectrum. As noted above, a reduced Raman cross section for the symmetric V⋯O stretching mode upon ^{18}O labeling is expected, and protein bands in the difference spectrum have comparable intensities. However, the band at 848 cm^{-1} in this difference spectrum is consistent with the expected isotopic frequency decrease of about 45 cm^{-1} in the symmetric V⋯O stretching mode observed in $(UVO_2)_2^{2-}$. The broad band in Figure 2a, at about 913 cm^{-1}, can be assigned to the asymmetric V⋯O stretching mode on the basis of its large intensity ratio of about 0.7 parallel to perpendicular incident light (inset below Figure 2a), and its moderate shift upon ^{18}O labeling of about 23 cm^{-1} (inset below Figure 2b). These assignments are confirmed in the RNase/UVO_2/H_2O ^{16}O minus ^{18}O difference spectrum, which is shown in the insert just above panel a of Figure 2. In this "isotope edited" difference spectrum, the protein bands subtract out, and the 893/848 cm^{-1} set of bands arising from the $^{16}O/^{18}O$ symmetric stretches are evident as is the ^{16}O asymmetric stretch at 913 cm^{-1}. The asymmetric ^{18}O band cannot be discerned because of its overlap with the ^{16}O symmetric stretch. Thus, when vanadate binds to the RNase/UVO_2/H_2O complex, the symmetric V⋯O stretching mode shifts down by 41 cm^{-1} while the asymmetric stretch mode is unchanged.

Our normal mode calculations[14] show that the V⋯O force constant *and* geometric factors, such as the angle between the two nonbridging V⋯O bonds, are important in determining the difference between the symmetric and asymmetric V⋯O stretch frequencies. For example, a reduction of the V⋯O stretch force constant of the two V⋯O bonds, coupled with an increased angle between the two bonds, can cause a downshift of the symmetric stretch mode while keeping the asymmetric stretch mode unchanged. Therefore, the fundamental frequency, which is the geometric average of the symmetric and asymmetric (see below), is the appropriate parameter to use in calculating bond lengths in a bond length/bond frequency relationship. It is related to bond force constant directly and is not affected by changes in geometry to a good approximation.[14]

Treatment of Frequency Data: Bond Length/Bond Strength/Vibrational Frequency Relationships. Accurate structural information about bonding in vanadate molecules (and often other metal and nonmetal oxides) can be obtained from vibrational spectroscopy by using two types of empirical relationships. One is the bond length/bond strength correlation pioneered by Brown and co-workers;[20,21] the other is the bond strength/vibrational frequency correlation formulated by Hardcastle and Wachs.[15] In the Brown and Wu relationship, a network paradigm is used to define bond strength, *s*, in terms of the average number of electron pairs per bond, so that $\sum s$ for any atom in a crystalline compound is equal to the formal valence, expressed in terms of valence units (vu) of that atom. In the case of vanadate, where the valence of vanadium is 5, $\sum s_{VO}$ calculated from eq 1 is expected to be close to 5.0 vu, where r_{VO} is the measured bond length (in Å).

$$s_{VO} = (r_{VO}/1.791)^{-5.1} \quad (1)$$

Bond strength also is related to vibrational frequency, and an empirical strength/frequency relationship can be cast in the same form as the Brown and Wu length/strength equation. Parameters for such an expression have been evaluated that allow bond strengths to be calculated from vibrational frequencies in crystalline and solution oxyvanadium compounds.[15,12] The validity of the Hardcastle/Wachs relationship, eq 2, can be evaluated from the rather minimal scatter in their frequency/strength plot for the VO bonds in a number of different crystalline oxyvanadium compounds where strengths/frequencies vary widely.[15]

$$s_{VO} = [0.2912 \ln(21349/\nu)]^{-5.1} \quad (2)$$

Initially, it was unclear what frequency should be used in eq 2, the symmetric stretch, ν_s, the asymmetric stretch, ν_a, or the "fundamental" frequency, ν, as defined by $[(\nu_s^2 + d\nu_a^2)/(d + 1)]^{1/2}$ (d is the degeneracy of the asymmetric modes). Now we have shown[14] that the fundamental frequency depends directly on the stretching force constant of the MO bond and is affected to a much smaller extent by geometrical and bond−bond coupling terms than is either ν_s or ν_a. Thus, ν provides a more

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(21) Brown, I. D. *Acta Crystallogr.* **1992**, *B48*, 553−572.

precise bond strength/frequency relationship. Therefore, the fundamental frequency will be used to calculate the bond strength in this paper.

Within their data set, which contains a variety of different oxyvanadium compounds, Hardcastle and Wachs show that the correlation of eq 2 is accurate to within ±20 cm^{-1}.[15] We believe that this error, which arises from the scatter of the data points about the fit to eq 2, is due in part to their use of a mixed set of frequencies, ν_s and ν. In the case of vanadates, this translates, via eq 1, into a length uncertainity, ±0.01 Å. It is expected that application of eq 2 to calculate *changes* in bond lengths, for example, by binding to a protein binding site, yields a substantially smaller error.

The fundamental stretching frequency for the V···O bonds in $(UVO_2)_2^{2-}$, 923 cm^{-1} (ν_s = 934 cm^{-1}; ν_a = 912 cm^{-1}), indicates a strength of 1.576 vu (eq 2), which is equivalent to a V···O bond length of 1.638 Å (eq 1). The fundamental stretching frequency for the V···O bonds in the RNase/UVO_2/H_2O complex is 903 cm^{-1} (ν_s = 893 cm^{-1}; ν_a = 913 cm^{-1}), which corresponds to a bond strength of 1.521 vu and a bond length of 1.650 Å. Thus, the 20 cm^{-1} decrease in the fundamental frequency of the VO stretching mode of $(UVO_2)_2^{2-}$ on forming RNase/UVO_2/H_2O corresponds to a decrease in V···O bond strength only of 0.055 vu, or to an increase in bond length only of 0.012 Å.

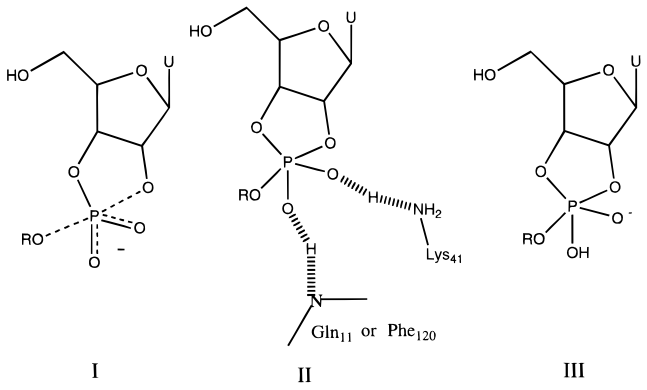
Discussion

The solution complex of RNase, uridine, and vanadate presumedly contains a pentacoordinate vanadium by analogy with the crystalline complex.[3,22] Since a water molecule bonds directly to the vanadium, we represent this complex as RNase/UVO_2/H_2O. There has been considerable interest in the structural details of this complex and solution vanadate complexes; a substantial effort has been expended in modeling[19] $(UVO_2)_2^{2-}$, and the structurally related adenosine/vanadate dimer,[18] $(AVO_2)_2^{2-}$.

In our investigation of the ⩾VO_2 unit in RNase/UVO_2/H_2O, we use known empirical relationships between bond strength, bond length, and the stretching frequency of nonbridging vanadium−oxygen bonds[14,15] (see Results). These relationships are essentially independent of both the geometry of a vanadate complex and the number and strength of its individual VO bonds. Bond strength and bond length obtained spectroscopically can be quite accurate with the accuracy depending on the accuracy with which empirical relationships correlate frequency with bond strength and bond length rather than on the actual frequency measurement. In the present case, the absolute accuracy of the empirical relationship is estimated to be better than about ±0.01 Å in bond length or ±0.05 vu in bond strength,[14,15] which is equivalent to an error of ±20 cm^{-1} for the stretching frequency. However, determinations of the differences in bond length of closely related compounds are expected to be limited primarily by the accuracy with which vibrational frequency differences can be measured. This is generally an order of magnitude smaller (ca. ±2 cm^{-1}).

The results of the current study show that the fundamental frequency (defined in Results) of the ⩾VO_2^- group in a solution of the uridine/vanadate dimer, $(UVO_2)_2^{2-}$, decreases by 20 cm^{-1} when this complex reacts with RNase to produce the RNase/UVO_2/H_2O complex. Thus, the strength of each of the vanadate−oxygen bonds of the ⩾VO_2^- groups (Chart 1) decreases by only 0.055 vu. This decrease corresponds to an increase in bond length only of about 0.012 Å and indicates a V···O bond length in the RNase/UVO_2/H_2O complex close to 1.650 Å.

Chart 2. Three Structures That Have Been Proposed for the Transition State of RNA Cleavage by Ribonucleases



This length does not agree well with the reported bond lengths for the ⩾VO_2^- group in the X-ray structure of the RNase/UVO_2/H_2O complex, 1.746 and 1.747 Å.[3,22] We suggest that the discrepancy of about 0.1 Å is caused by inaccuracies inherent in a protein structure where data extend to a Bragg spacing of only 2 Å and where the structure of the vanadate group was not refined because of the variability of VO bond lengths and geometry. In support of this suggestion, we also note the observation of Brown and Wu[20] that when the summed bond strengths for V(V) obtained by eq 1 lie outside the range of 5.0 ± 0.2 vu, an error in the bond length data is likely. The summed bond strength for the five VO bonds in the published structure of RNase/UVO_2/H_2O complex is only 4.5 vu. Thus, the smaller-than-normal value of $\sum s_{VO}$ suggests that some or all of the published VO bond lengths of RNase/UVO_2/H_2O are too long. The most likely error is an overestimate in the length of the V···O bonds of the ⩾VO_2^- group.

There have been various proposals for the structure of the transition state in the RNase catalyzed reaction, and these are represented in Chart 2. The structure most generally supported by various experiments (cf. ref 8) is based on an S_N2-like displacement (structure **I**, Chart 2), where the impetus for breaking the P−O bond of the leaving group is provided by increased bonding to the entering oxygen. The *sum* of the two axial bonds PO bond strengths involving the entering and leaving groups is approximately constant as the reaction proceeds. A constant value of $\sum s_{PO}$ for the bridging oxygens implies a constant value of $\sum s_{PO}$ for the nonbridging P···O bonds, since $\sum s_{PO}$ for *all* bonds in a phosphate ester is not expected to change significantly during hydrolytic cleavage.

Two other putative transition state structures have been proposed. One involves an addition/elimination process where collapse of a pentacoordinate phosphorane-like triester intermediate provides the impetus for bond breaking in the second step of the two-step reaction catalyzed by RNase (see Scheme 1). Protonation of the nonbridging oxygens to form the phosphorane-like adduct was suggested.[11] This transition state structure is pictured in structure **III** of Chart 2. In such a process, the strength of the nonbridging P···O bonds will decrease from about 1.37 vu, characteristic of a phosphate diester monoanion,[19] to a value close to 1.0 vu. Last, another mechanism, involving substantial nonbridging P−O bond

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polarization brought about by the formation of low-barrier hydrogen bonds or partial hydrogen transfer to the nonbridging oxygens in order to stabilize the pentacoordinate intermediate,[23] is shown in structure **II** of Chart 2.

As discussed in the Introduction, the transition state binding paradigm and other considerations suggest that the RNase/UVO_2/H_2O complex represents a structure that is about halfway (or more) along toward the transition state structure of the RNase catalyzed hydrolysis of uridine 2′,3′-cyclic phosphodiester. On this basis, a correspondence can be drawn between the changes that occur when $(UVO_2-OH_2)^-$ binds to RNase to form RNase/UVO_2/H_2O and progress along the reaction coordinate of RNase catalyzed hydrolysis of uridine 2′,3′-cyclic phosphate. The total bond strength of the two nonbridging P⋯O bonds relative to that of the ground state in the enzyme catalyzed reaction is reduced *only* by between twice 0.055 or 0.11 vu (if the enzymic vanadate complex is structurally the same as the transition state) and 0.22 vu (if this complex is halfway along to the path to the transition state) compared to the ground state. Thus, the sum of the axial bond strengths (the O−P−OR bonds in Chart 2) should increase by 0.11 to 0.22 vu in the transition state compared with the ground state since the loss in bond strength of the nonbridging P⋯O bonds will be transferred to these axial PO bonds in order to maintain a constant valence bond order around the phosphorus atom (see Results). Hence the nonbridging P⋯O bonds undergo only very small bond polarization compared to the ground state, and the transition state resembles structure **I** of Chart 2. We conclude from this that the reaction mechanism is mostly S_N2-like, but with a small associative charactor as deduced from the small increase in axial bond order. The putative transition state structures shown in **II** and **III** of Chart 2 are excluded although the following caveat applies to the notion of low barrier (high energy) hydrogen bond formation at the binding site. As discussed in Results, the Brown and Wu relationships hold that the sum of the bond strength about an atom is equal to its valence. The valence of oxygen is two and the nonbridging V⋯O bond order is about 1.5 vu for the model vanadate complex in solution, $(UVO_2)_2^{2-}$, and in the transition state analogue RNase/UVO_2/H_2O complex (see Results). Hence, the oxygen atom of the nonbridging V⋯O bonds is estimated to form a hydrogen bond or bonds that total 0.5 vu in solution and in the RNase. This is quite large. In water, two or more water molecules can donate relatively low energy H bonds. However, if there is only one hydrogen bond donor group at the RNase binding site, as suggested by the crystallographic structure of RNase/UVO_2/H_2O,[3] this single hydrogen bond would have to be considered relatively strong.

It is interesting to note that a recent study based on the ^{18}O isotope effects of the RNase catalyzed reaction is in excellent quatitative agreement with our conclusions, since it suggests that 0.13−0.20 vu is lost to the nonbridging oxygen atoms in the transition state of the chain cleavage step.[10] Although our estimated total loss of the bond strength to the nonbridging P⋯O bonds in the transition state, 0.11−0.22 vu, refers to the hydrolysis of the 2′,3′-cyclic phosphate, which is the second step of the RNase catalyzed reaction, the agreement between these two studies on the reaction mechanism is striking because it is generally assumed that RNase employs a similar mechanism in both reaction steps.[10]

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