In the case of peptide hydrolyses catalyzed by carboxypeptidase A it has been suggested that the phenolic hydroxyl of Tyr-248 may function to donate a proton to help generate the incipient leaving amino group.[2] Evidence has been obtained that for the hydrolysis of reactive esters catalyzed by CPA the corresponding donation of a proton from the Tyr-248 hydroxyl to aid formation of the leaving alcohol derived from the substrate is not important.[8] When the geometry of the ketonic substrate (−)-**1** bound as pictured in Figure 3 is considered, it can be seen that the tyrosine hydroxyl group cannot be functioning in its phenoxide form as the proton-abstracting group or in its phenolic form as the proton donor because this would predict exchange at the H_b (*pro-S*) position of the 3-methylene group.

In considering mechanisms of reaction at the active sites of hydrolytic enzymes, the problem is frequently faced whether the catalytic apparatus consists of functional groups in the arrangement A–H- -B or as A- -H–B, in other words, whether the acidic group is A-H and the basic group is B or the reverse holds. Similarly, if the trapping of an anhydride species in the CPA-catalyzed hydrolysis of the reactive ester *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate[9] were considered to be a special case, then there is a possibility (although it seems unlikely to us) that the binding of (−)-**1** to the active site region of CPA might be taking place according to the picture shown in Figure 4. If this were the situation, then the abstracting base would be zinc hydroxide and the carbonyl group of the substrate would be held in place by the carboxyl function of Glu-270. While several aspects of such a scheme are not attractive, it is hard to rule out on purely kinetic grounds. However, our stereochemical results clearly show that the CPA-catalyzed enolization reaction cannot be taking place with the substrate bound in this geometry. In particular, if zinc hydroxide were to remove the proton at the 3-methylene position of (−)-**1**, the hydrogen which would undergo the exchange reaction would be at the H_b (*pro-S*) position, contrary to our stereochemical observations. One last possibility is that (−)-**1** binds as shown in Figure 4 using the carboxyl of Glu-270 to hold the substrate's carbonyl group and that the phenoxide form of Tyr-248 acts as the abstracting base and the phenolic form of this residue as the proton donor. However, this scheme would lead to the prediction that k_{cat} would show a bell-shaped pH dependency, whereas in fact we have observed a sigmoidal dependency with no evidence that ionization of Tyr-248 plays any kinetically significant role.[10]

In summary, we have demonstrated that it is the *R* isomer of **1** which undergoes CPA-catalyzed hydrogen–deuterium exchange at its 3-methylene group and that it is the *pro-R* hydrogen which is susceptible to exchange at that position.

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Role of Active-Site Residues in the Catalytic Mechanism of Ribonuclease A

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Abstract: The electronic roles of His-12, Lys-41, Asn-44, His-119, the 119–120 backbone N–H, and Asp-121 in the transphosphorylation stage of the RNase A mechanism are assigned by combining known experimental facts with electronic structure theory calculations. His-12 is more likely to deprotonate $O_{2'}$, but in its absence Asn-44 may fulfill this role; His-119 activates the leaving group and facilitates in-line addition of $O_{2'}$; the 119–120 backbone N–H increases the electrophilicity of phosphorus; Gln-11···H_2O functions in a manner similar to the 119–120 backbone N–H; Lys-41 increases the phosphorus electrophilicity and stabilizes the trigonal-bipyramidal intermediate; Asp-121 may position the substrate, but does not act as a charge relay with His-119.

Although the catalytic mechanism of ribonuclease A (RNase A) has been studied extensively, uncertainty remains about various aspects. In this paper we attempt a description of the electronic rearrangements attendant to catalysis by combining the known experimental facts (including some unpublished X-ray data[1-3]) with results from electronic structure calculations. RNase A catalyzes the hydrolysis of a ribonucleic acid or a nucleotide ester by a two-stage mechanism.[4] The first stage, transphosphorylation,[5] involves addition of the 2′-OH group (on the 3′-ribose) to the phosphate group cleaving the ribonucleic acid chain at the 5′ end yielding a 2′-3′ cyclic phosphate and a free 5′-OH group (Figure 1). The second stage, hydrolysis,[5] involves addition of H_2O to the cyclic intermediate, yielding a terminal 3′-phosphate monoester (Figure 1). We are concerned primarily with stage one in the present article.

Viewed in its simplest terms, the transphosphorylation stage can be divided into several distinct steps.[4,6,7] In actuality it is likely that steps 2–5 are concerted. (1) Substrate is bound to the enzyme and the substrate–amino acid residue interactions prepare it for 2′-OH addition by making the phosphorus more electrophilic. (2) The 2′-OH group on the 3′-ribose is at least partially deprotonated, making it a better nucleophile. (3) During or after deprotonation, the phosphate group rotates

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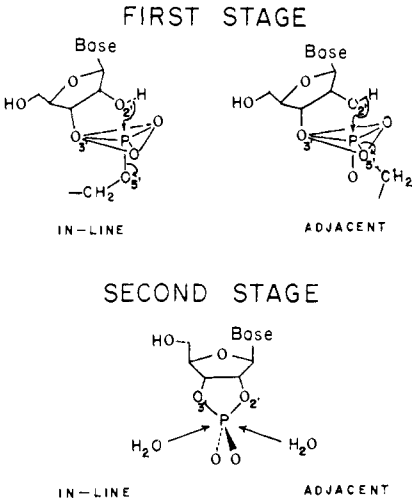


Figure 1. In-line and adjacent mechanisms for the first (transphosphorylation) and second (hydrolysis) stages of the RNase A catalytic mechanism.

toward the 2′-O⁻. (4) The 2′-O⁻ adds to the phosphate group forming a trigonal-bypyramidal (TBP) transition state or intermediate. Usher et al.[8] and Eckstein et al.[9] have given evidence that this nucleophilic addition proceeds by an in-line mechanism,[10a] i.e., an S_N2-like reaction whereby the TBP formed has the 2′-O⁻ and 5′-O leaving group in apical positions.[10b] (5) The TBP breaks down aided by protonation of the 5′-O to form free 5′-ROH and a 2′–3′ cyclic intermediate.[10c] The transphosphorylation reaction is summarized schematically in Figure 2. The amino acids believed to be involved in the reaction are included in the figure.

Kinetic studies using the temperature-jump method[6] indicate that there are three activity-linked groups at the active site of RNase A with pK values of approximately 5, 6, and 6.7. Two of these pKs are believed to correspond to His-12 and His-119.[4,6,7] The third has been associated with either Asp-121[11-13] or His-48.[4,6] Additional active-site groups postulated to fulfill the roles mentioned above are Lys-41[14-16] and the backbone N–H between His-119 and Phe-120.[1] With the exception of His-48, we have investigated all of the amino acids implicated above and have assigned a role to each.

Our principal attention has been focused on three of the five transphosphorylation steps noted above: attachment and partial protonation (step 1), deprotonation (step 2), and nucleophilic addition (step 4).

Methods

Charge distributions (including overlap populations), geometries, and relative energies are obtained from semiempirical (CNDO/2) electronic structure theory calculations and employed as a qualitative aid to chemical reasoning. In particular, the results of these numerical experiments have been used to help clarify the roles of His-119, the 119–120 N–H backbone, and Asp-121 in the activation of the phosphate group prior to nucleophilic addition and TBP formation. The functions of Lys-41, His-119, and Asp-121 after the 2′-OH has been deprotonated and is just beginning to add to the phosphate group are also brought out by the computed data.

The CNDO/2 scheme[17] has been utilized extensively and its capabilities and shortcomings are well established.[18,19] In general, covalent bonds are predicted to be too short, bond energies are predicted to be too large, and atomic charges are exaggerated. Hydrogen bond lengths are represented with moderate to good accuracy. Relative charge distributions (especially if group charges rather than atomic charges are computed) for molecular sequences are reproduced correctly.

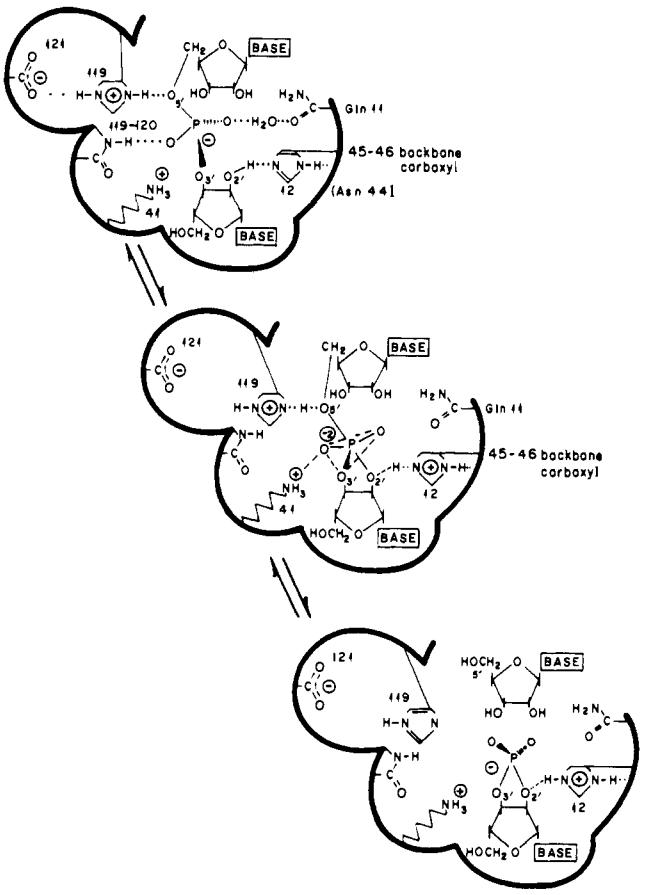


Figure 2. Schematic representation of the transphosphorylation reaction of RNase A.

Specific assessment of CNDO/2 reliability for the present study was obtained by comparing CNDO/2 and ab initio results for phosphine oxide[20,21] and dimethyl phosphate monoanion.[21,22] The CNDO/2 calculations were carried out for phosphine oxide both with and without 3d orbitals on P. The results show (Table I): (1) Ab initio and CNDO/2 results agree more closely when d orbitals are not included in the CNDO/2 basis set. Consequently, we have carried out all of the calculations reported in this paper without d orbitals on P. (2) Although the absolute magnitudes of the atomic charges are not very well reproduced by CNDO/2, the charge separation in a bond and the relative magnitudes of the charges are reasonably well reproduced. The important differences in the phosphorus–oxygen charge separation and the charges on the oxygens are reproduced correctly for the two types of oxygen in dimethyl phosphate monoanion. (3) The CNDO/2 P–O overlap populations parallel the ab initio overlaps.

Solvation and dielectric effects are an important aspect of enzymatic processes and several recent articles have made significant contributions to this problem.[23] For that part of the RNase A mechanism studied here, individual water molecules enter the catalysis in two places: (1) Asp-121···H_2O···5′-base ring (adenine ring); (2) Gln-11···H_2O···phosphoryl oxygen.[4] In the first case a water molecule acts as a proton donor for a strong negative ion hydrogen bond to Asp-121 and as a proton donor for a normal hydrogen bond to nitrogen on the substrate adenine ring. In the second case a water molecule hydrogen is linked to Gln-11 by a normal hydrogen bond and the other hydrogen is involved in a medium-strong hydrogen bond to a phosphoryl oxygen of the substrate. Previous research on coupled hydrogen bonds of this type[24] has provided enough information about their properties so that their role in RNase

Table I. Atomic Charges and P-O Overlap Populations for Phosphine Oxide and Dimethyl Phosphate Monoanion

molecule	basis set	P atomic charge	O atomic charge	H/CH_3 atomic charge	P-O overlap population
phosphine oxide	GTO[a]	1.08	−0.72	−0.12	1.39
$[POH_3]$	4-31G[b]	0.99	−1.01	+0.01	0.35
	CNDO/2 (no d orbitals on p)[b]	0.77	−0.45	−0.11	1.18
	CNDO/2 (d orbitals on p)[b]	0.44	−0.24	−0.07	2.59
dimethyl phosphate monoanion	STO-3G[c]	1.38	−0.48 (−0.74)[d]	+0.03	0.488 (0.256)[d]
$[PO_4C_2H_6]^-$	CNDO/2 (no d orbitals on p)[c]	1.19	−0.46 (−0.61)[d]	−0.02	1.06 (0.905)[d]

[a] Reference 20. [b] Reference 21. [c] Reference 22. [d] The number in parentheses refers to the phosphoryl oxygen.

A can be satisfactorily characterized without further detailed computation.

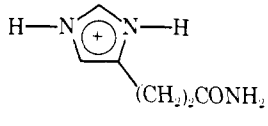
Solvent molecules and those residues which form the active-site pocket provide a dielectric medium that reduces the strength of the interaction between charged residues on the enzyme (His-119, Asp-121, Lys-41) and the negatively charged phosphate group of the substrate. The qualitative effects of dielectrics can be understood from the well-known solutions of Poisson's and Laplace's equations for simple cases.[25a] Thus our calculations exaggerate interactions between charged species, e.g., Lys-41 and the phosphate group. Since the precise location of Lys-41 and whether or not there are water molecules between it and other residues of the enzyme is not known, a more detailed treatment of dielectric effects is not worthwhile at present. The qualitative trends in the active-site residue–substrate interactions are given correctly by the present computations.[25b]

There are additional interactions for which sufficiently accurate structural information is lacking or quantitative calculations are impractical, e.g., the base stacking between His-119 and the 5′-base ring of substrate. Our overall method has been to combine experimental results with approximate electronic structure calculations for the purpose of assigning consistent and chemically reasonable electronic roles to residues in the active site.

Models

A computational approach can treat particular parts of the enzyme separately, thereby aiding in the elucidation of their function. Thus, our strategy is to treat individual residues and successively larger combinations of them, in each instance determining the atomic charges, overlap populations, relative energetics, and optimized geometries.

Attachment and Partial Protonation Step of Transphosphorylation. Since charge transfer through the main chain is expected to be minimal, His-119, Asp-121, and the 119-120 backbone N-H are represented by protonated imidazole,[26] formate ion, and formamide, respectively. The model for the substrate is dimethyl phosphate monoanion, $[PO_4C_2H_6]^-$.[28] A second, connected model for the His-119-backbone N-H system was also investigated:



Calculations with and without the His-119-backbone N-H covalent connection were carried out. The resulting charge distributions, overlap populations, and hydrogen bond energies were identical, thereby supporting the hypothesis of negligible charge transfer through the main chain.

$[PO_4C_2H_6]^-$, $PO_4C_2H_7$ (protonated methoxy group), and each of the active-site species are calculated individually for reference. The bond lengths, bond angles, and dihedral angles used for defining the substrate geometry are taken from the X-ray crystal-structure values for uridyl-3′,5′-adenosine phosphate (UpA).[29]

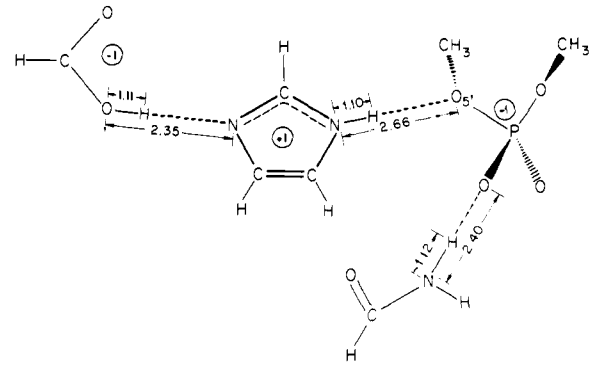


Figure 3. Fully connected model for the attachment and partial protonation stage of transphosphorylation. Bond lengths are energy optimized values. Approximate formal starting charges are shown, but the hydrogens are in the optimized positions. (Note that the 119-120 backbone N-H, $HCONH_2$, pictured in the plane of the page, is actually in the OPO phosphoryl oxygen plane perpendicular to the page.)

In accordance with the X-ray structure of UpA,[29] we use an $O_{3'}PO_{5'}C_{5'}$ dihedral angle (ω) of 271° and $C_{3'}O_{3'}PO_{5'}$ dihedral angle (ω') of 164°. A recent theoretical calculation by Gorenstein et al.[31a-c] suggests that a better choice may be $\omega = 60°$, $\omega' = 180°$. Nevertheless, it is clear from our results that although the substrate conformation may change the degree to which substrate-active-site residue interactions affect substrate properties, the trends in these property changes will remain the same.

The N-H bond lengths in protonated imidazole were obtained by energy optimization. Experimental values were employed for all the other bond lengths and bond angles in imidazole as well as for the formate ion and formamide.[30]

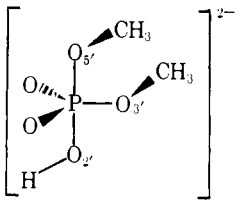
Hydrogen bonds between Asp-121 and His-119, His-119 and substrate, and 119-120 backbone N-H and substrate have been postulated.[32,33] Thus, we examined the following combinations: His-119···sub, sub···N-H (back), His-119···sub···N-H(back), Asp-121···His-119, Asp-121···His-119···sub, Asp-121···His-119···sub···N-H(back). In each hydrogen bond link, the distance between the heavy atoms and the position of the proton were determined by energy optimization.[34a] The fully connected combination with the internuclear separations calculated is shown in Figure 3.

Nucleophilic Addition (or Cyclization Step) of Transphosphorylation. The amino acids postulated to be involved in the addition of 2′-O^- to the tetrahedral phosphate group are Lys-41, His-119, and Asp-121. The models for the latter two are the same as above. The model for Lys-41 is NH_4^+. The substrate is represented by TBPI (shown below). We have assumed in-line, apical addition of the incoming nucleophile[8,9] and an ideal trigonal-bipyramidal transition state. The conformation of TBPI used in the calculations is essentially the g,t,t conformation found by Gorenstein et al.[31d,e] to be pre-

Table II. Total Energies and Hydrogen Bond Energies (Kilocalories/Mole) for the Attachment and Partial Protonation Step of Transphosphorylation

species	total energy	H-bond energy
imidazole$^+$ (His)	−30 209.2	
$HCOO^-$ (Asp)	−27 771.0	
H_2NCOH(NH(back))	−24 674.2	
$[PO_4C_2H_6]^-$(sub)	−61 300.0	
His-NH(back)	−54 884.2	
His-sub	−91 637.9	−128.6
sub-NH(back)	−86 006.1	−31.9
His-sub-NH(back)	−116 323.7	−140.3
Asp-His	−58 117.6	−197.5
Asp-His-sub	−119 503.1	−222.9
Asp-His-NH(back)	−82 848.8	
Asp-His-sub-NH(back)	−144 196.3	−242.0
His-sub(H bond to phosphoryl oxygen)	−91 676.2	−167.0

ferred for OH^- attack. For simplicity, we have also assumed that $O_{2'}$ is totally deprotonated, but it is likely that the deprotonation of $O_{2'}$, rotation of the phosphate group, and nucleophilic additon of $O_{2'}$ are concerted. This assumption will not affect the trends in the changes in substrate properties found from our calculations. Therefore, the conclusions drawn remain the same.[34b]



TBPI

Only the phosphorus-oxygen bond lengths differ from those in the tetrahedral phosphate above. The basal P-O bonds have been lengthened slightly (by 0.03 Å) in accordance with recent results on phosphine oxides.[35] The P-$O_{2'}$ bond length was chosen as 2.5 Å because our calculations show that the two groups have clearly begun to interact at this distance. P-$O_{5'}$ was optimized at 2.10 Å. The standard value of 1.0 Å is taken for O-H. N-H in NH_4^+ is assumed to be 1.012 Å.[36]

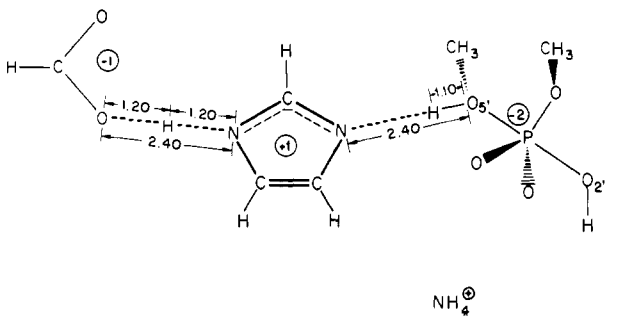


Figure 4. Fully connected model for the cyclization stage of transphosphorylation. Energy-optimized bond lengths. Approximate formal starting charges are shown, but the hydrogens are in the final optimized positions. The substrate is shown in the TBP geometry with the phosphate group positioned to represent the N···H hydrogen bond angle.

TBPI, TBPI with $O_{5'}$ protonated, and each of the active-site species were computed for reference. Calculations were carried out on the same combinations listed in the section above but with the backbone N-H replaced by Lys-41. The Asp-121···His-119 and the His-119···TBPI heavy atom and proton positions were optimized, but the TBPI···Lys-41 positions were not. The position of Lys-41 with respect to TBPI is uncertain, and it is not known whether Lys-41 is within hydrogen bonding distance of TBPI.[4] Consequently, for each combination containing Lys-41, calculations were made with Lys-41 at two different distances from TBPI, 2.5 and 4 Å. The fully connected system with the computed internuclear separations is given in Figure 4.

Results and Discussion

Attachment and Partial Protonation. His-119. The crystal structure of the substrate analogue UpcA bound to RNase A[1] indicates that there is a hydrogen bond between His-119 and $O_{5'}$ of the substrate (see Figure 2). This His-119-substrate interaction will influence the charges on P and $O_{5'}$ and the P-$O_{5'}$ bond strength. Table II (entry 6) shows that the His-119-$O_{5'}$ hydrogen bond is very strong with the proton approximately halfway between them (Table III, entry 1), i.e.,

Table III. Geometries of Active-Site Models for Attachment and Partial Protonation Step of Transphosphorylation[a] (H-Bond Distances in Å)

species	Asp-His H bond	His-sub[b] H bond	sub[b]-NH(back) H bond
His-sub(H bond to $O_{5'}$)		←2.44→ N···H···O 1.26 1.18	
sub-NH(back)			←2.43→ O·····H—N 1.15
His-sub-NH(back)		←2.44→ N····H····O 1.25 1.19	←2.46→ O·····H—N 1.12
Asp-His	← 2.47 → O—H·····N 1.08		
Asp-His-sub	← 2.41 → O—H·····N 1.12	← 2.53 → N—H·····O 1.12	
Asp-His-sub-NH(back)	← 2.35 → O—H·····N 1.11	← 2.66 → N—H····O 1.10	←2.40→ O·····H—N 1.12
His-sub(H bond to phosphoryl oxygen)		← 2.47 → N·····H—O 1.09	

[a] All H bond distances are optimized. [b] Sub is $[PO_4C_2H_6]^-$ for all entries.

Table IV. Overlap Populations for Attachment and Partial Protonation Step of Transphosphorylation

species	P-$O_{5'}$	$O_{5'}$-H
$[PO_4C_2H_6]^-$(sub)	0.904	
$PO_4C_2H_7$($O_{5'}$ protonated)	0.643	0.803
His-sub(H bond to $O_{5'}$)	0.743	0.557
sub-NH(back)	0.931	
His-sub-NH(back)	0.773	0.510
Asp-His-sub	0.843	0.214
Asp-His-sub-NH(back)	0.889	0.123
His-sub(H bond to phosphoryl oxygen)	0.997	

Table V. Atomic Charges for Attachment and Partial Protonation Step of Transphosphorylation (in Atomic Units)

species	P	$O_{5'}$	$O_{5'}CH_3$
$[PO_4C_2H_6]^-$(sub)	1.24	−0.47	−0.49
$PO_4C_2H_7$($O_{5'}$ protonated)	1.16	−0.24	−0.02
His-sub(H bond to $O_{5'}$)	1.21	−0.40	−0.29
sub-NH(back)	1.29	−0.45	−0.45
His-sub-NH(back)	1.25	−0.41	−0.30
Asp-His-sub	1.23	−0.47	−0.46
Asp-His-sub-NH(back)	1.27	−0.47	−0.45
His-sub(H bond to phosphoryl oxygen)	1.36	−0.41	−0.34

the substrate is partially protonated. As a consequence, the P-$O_{5'}$ bond is weakened[37] (Table IV, entries 1 and 3), the positive charge on P is decreased (making P less electrophilic), and the negative charge on $O_{5'}$ and the $O_{5'}CH_3$ group is decreased (Table V, entries 1 and 3). Thus, the role of His-119 is to make the P-$O_{5'}$ bond more reactive and $O_{5'}$($O_{5'}CH_3$) more electronegative. This increases its preference for an apical position when $O_{2'}$ attacks and is consistent with an in-line mechanism. In addition, His-119 delocalizes the negative charge on the anionic phosphate group (Table VI, entry 1).

119-120 Backbone N-H. The X-ray data[1] indicate a hydrogen bond between the 119-120 backbone N-H and a phosphoryl oxygen, leading to the possibility that the N-H hydrogen bond increases the electrophilicity of P and stabilizes the pentacoordinate intermediate (see Figure 2). Our results (Table II, entry 7, and Table III, entry 2) show that the hydrogen bond between these two groups is of medium strength (estimated to be 10-15 kcal/mol) with the hydrogen remaining on the backbone nitrogen, i.e., the phosphoryl oxygen is not protonated. This interaction produces a more positive (electrophilic) P, a slightly less negative $O_{5'}$ and $O_{5'}CH_3$ (Table V, entries 1 and 4), and a stronger P-$O_{5'}$ bond (Table IV, entries 1 and 4). It also slightly delocalizes the negative charge on the substrate (Table VI, entry 2).

The proposed Gln-11···H_2O···phosphoryl oxygen (second phosphoryl oxygen) hydrogen bonds[4] (see Figure 2) are expected to have a function similar to that described above for the 119-120 N-H system, since the hydrogen bonds in the Gln-11 system are also of medium strength.[24]

A hydrogen bond between His-119 and a phosphoryl oxygen has also been mentioned as a possibility in the literature.[47] The X-ray data[1] and the above theoretical results for the role of the backbone N-H make this possibility unlikely. A His-119-phosphoryl oxygen hydrogen bond would be very strong (Table II, entry 13) with the phosphoryl oxygen protonated (Table III, entry 7).[38] Although a protonated phosphoryl oxygen would further increase the electrophilicity of P (Table V, entries 1 and 8), it would also make the P-$O_{5'}$ bond considerably stronger than in the isolated substrate (Table IV, entries 1 and 8). Since P in the isolated substrate already bears a very high positive charge (Table V, entry 1), it would seem to be more important to activate the leaving group than to produce a more electrophilic P, especially if the transphosphorylation reaction is concerted. Consequently, a medium strength hydrogen bond to a phosphoryl oxygen is preferable to a strong one, since it brings about the same change in substrate but to a lesser extent. Two additional reasons for preferring a weaker hydrogen bond (and a nonprotonated phosphoryl oxygen) are: (1) the in-line addition of $O_{2'}$ will be enhanced if the phosphoryl oxygens are not protonated, since these oxygens are then not likely to occupy an apical position when $O_{2'}$ attacks the tetrahedral phosphate;[10a] (2) less energy will be required to rotate the substrate in preparation for $O_{2'}$ addition, since less energy is required to break a medium strength phosphoryl oxygen hydrogen bond.

A recent NMR experiment has been interpreted as indicating an interaction between Lys-41 and a phosphoryl oxygen in the attachment step.[40] This is in conflict with crystallographic results[1,2,4,14] that show Lys-41 and phosphate separated by a large distance when the substrate is first bound and with our results that suggest that the interaction will be quite weak if it exists. If a strong hydrogen bond were formed between Lys-41 and a phosphoryl oxygen, it would produce the same unfavorable effects described above for a His-119-phosphoryl oxygen interaction. It has also been suggested that Arg-10 rather than Gln-11 interacts with one of the phosphoryl oxygens.[41] We feel this suggestion is unlikely for the reasons cited above.

The His-119···Sub···N-H(Back) Combination. We have shown that a N-H(back)-substrate interaction increases the P electrophilicity but strengthens the P-$O_{5'}$ bond, while a His-119-substrate interaction weakens the P-$O_{5'}$ bond but decreases the P electrophilicity. It would obviously be preferable to have a substrate-active site interaction that would activate both P and the leaving group. Our results show that this is realized by the His-119···sub···N-H(back) combination (see Figure 2). Compared to the isolated substrate, P is more positive, $O_{5'}$($O_{5'}CH_3$) is less negative (Table V, entries 1 and 5), the P-$O_{5'}$ bond is weaker (Table IV, entries 1 and 5), negative charge is delocalized (Table VI, entry 3), and the substrate has been stabilized (Table VII, entry 2).

Asp-121. Asp-121 is believed to interact with His-119, most

Table VI. Charges per Molecular Unit for Attachment and Partial Protonation Step of Transphosphorylation (in Atomic Units)

species	charge	His-119	sub[a]	119-120 NH(back)	Asp-121
His-sub[b](H bond to $O_{5'}$)	0	+0.41	−0.41		
sub-NH(back)	−1		−0.71	−0.29	
His-sub-NH(back)[b]	0	+0.46	−0.34	−0.12	
Asp-His	0	+0.12			−0.12
Asp-His-sub	−1	+0.09	−0.88		−0.21
Asp-His-sub-NH(back)	−1	+0.16	−0.77	−0.17	−0.22

[a] Sub is $[PO_4C_2H_6]^-$. [b] The proton charge in the hydrogen bond between His-119 and the substrate has been divided evenly between them.

Table VII. Substrate Stabilization for Attachment and Partial Protonation Step of Transphosphorylation

species	ΔE^a
sub{$[PO_4C_2H_6]^-$}	0
His-sub-NH(back)	−139.4
Asp-His-sub	−25.1
Asp-His-sub-NH(back)	−47.7

[a] ΔE = energy of model RNase A active site plus substrate − (energy of RNase active-site model + energy of isolated substrate), e.g., energy of optimized Asp-His-sub minus energy of optimized Asp-His minus energy of isolated substrate.

Table VIII. Total Energies and Hydrogen Bond Energies (in Kilocalories/Mole) for Cyclization Step of Transphosphorylation

species	total energy	H-bonded energy
$[PO_5C_2H_7]^{2-}$(sub)	−73 308.2	
imidazole$^+$(His)	−30 209.2	
$HCOO^-$(Asp)	−27 771.0	
H_4N^+(Lys)	−8 998.8	
His-sub	−103 783.6	−266.2
sub-Lys[a]	−82 424.9	
His-Lys	−39 092.4	
His-sub-Lys[a]	−112 846.4	−330.2
Asp-His	−58 117.6	−197.5
Asp-His-sub	−131 577.9	−289.4
Asp-His-Lys	−67 174.8	
Asp-His-sub-Lys[a]	−140 676.8	−389.6

[a] Lys-41 is 4.0 Å from the substrate.

Table IX. Geometries of Active-Site Models for Cyclization Step of Transphosphorylation[a] (H-Bond Distances in Angstroms)

species	Asp-121-His-119	His-119-sub[b]
His-sub		← 2.52 → N·····H—O 1.06
Asp-His	← 2.47 → O—H·····N 1.08	
Asp-His-sub	← 2.40 → O·····H·····N 1.20	← 2.40 → N·····H—O 1.10

[a] All H-bond distances are optimized. [b] Sub is $[PO_5C_2H_7]^{2-}$ for all entries.

likely via a hydrogen bond,[1,2,4,14,15,42] and this interaction is thought to be important in controlling the pK of His-119 (see Figure 2).[11-13]

In order to explore the role of Asp-121, calculations with optimized geometries were carried out for His-119···sub, Asp-121···His-119···sub, His-119···sub···N-H(back), Asp-121···His-119···sub···N-H(back), and Asp-121···His-119. When Asp-121 is added to the His-119···sub or His-119···sub···N-H(back) systems, His-119 interacts so strongly with Asp-121 that the His-119-substrate interaction is weakened considerably. This produces the following results: (1) There is proton transfer from the substrate to His-119 and from His-119 to Asp-121, i.e., the substrate is no longer partially protonated (Table III, entries 5 and 6). (2) The charge on $O_{5'}$ ($O_{5'}CH_3$) (Table V, entries 1, 6, and 7) and the P-$O_{5'}$ overlap population (Table IV, entries 1, 6, and 7) revert to their values in the isolated substrate with the charge on P changed only slightly. (3) The delocalization of substrate negative charge is reduced (Table VI, entries 5 and 6). (4) Stabilization of the substrate is reduced (Table VII, entries 3 and 4). The net effect is that the substrate remains essentially unchanged. We conclude that a strong interaction between Asp-121 and His-119 is unlikely and that Asp-121 has little importance in this part of the catalytic mechanism other than electrostatic stabilization of the positive charge on His-119. This conclusion is supported by the large His-119-Asp-121 separation (3.6 Å) found from crystallographic studies.[2] By contrast, the His-Asp separation in α-chymotrypsin, where there is known to be a strong interaction, is 2.8 Å.[43,44] In view of the widespread discussion of the charge relay in serine proteases, it is worth pointing out the dissimilarity between α-chymotrypsin and RNase A. In α-chymotrypsin the Asp-102-His-57 charge relay transfers negative charge *to* the substrate; in RNase A it is necessary to transfer negative charge *from* the substrate. Thus Asp-121 involvement is disadvantageous, since its negative charge would diminish the charge transfer from the substrate (Table VI, entries 5 and 6).

Table X. Overlap Populations for Cyclization Step of Transphosphorylation

species	P-$O_{5'}$	P-$O_{2'}$	$O_{5'}$-H
$[PO_5C_2H_7]^{2-}$(sub)	0.317	0.120	
$[PO_5C_2H_8]^{1-}$($O_{5'}$ protonated)	0.174	0.164	0.892
His-sub	0.187	0.159	0.892
sub-Lys[a]	0.372	0.153	
His-sub-Lys[a]	0.211	0.194	0.881
Asp-His-sub	0.214	0.146	0.763
Asp-His-sub-Lys[a]	0.250	0.181	0.731

[a] Lys-41 is 2.5 Å from the substrate.

Table XI. Atomic Charges for Cyclization Step of Transphosphorylation (in Atomic Units)

species	P	$O_{5'}$	$HO_{5'}CH_3$
$[PO_5C_2H_7]^{2-}$(sub)	1.21	−0.57	−0.78[b]
$[PO_5C_2H_8]^{1-}$($O_{5'}$ protonated)	1.11	−0.23	+0.05
His-sub	1.13	−0.32	−0.01
sub-Lys[a]	1.37	−0.53	
His-sub-Lys[a]	1.29	−0.36	−0.02
Asp-His-sub	1.17	−0.40	−0.11
Asp-His-sub-Lys[a]	1.34	−0.44	−0.13

[a] Lys-41 is 2.5 Å from the substrate. [b] Charge on $O_{5'}CH_3$.

Studies carried out on RNase A derivatives show that an acidic group is needed at position 121, and Carlson's crystallographic data[1] indicate the possibility of an Asp-121···H_2O···5′-base (adenine) hydrogen-bonded system. These results suggest another role for Asp-121: it positions the adenine ring and this ring in turn aligns His-119 in site IV via base stacking.[1] The interposed water molecule is necessary to reduce the net interaction to that of a normal hydrogen bond.[45] If Asp-121 were hydrogen bonded directly to the adenine ring, the bond would be very strong and this would be unfavorable since it must break when the substrate rotates.[24]

Deprotonation and Nucleophilic Addition (or Cyclization Step) of Transphosphorylation. His-12. The nucleophilic addition of $O_{2'}$ to the phosphate group will be facilitated by at least partial deprotonation of the 2′-OH group (see Figure 2). His-12 has been suggested as the group that carries out this deprotonation.[6,7,27b,46,47a]

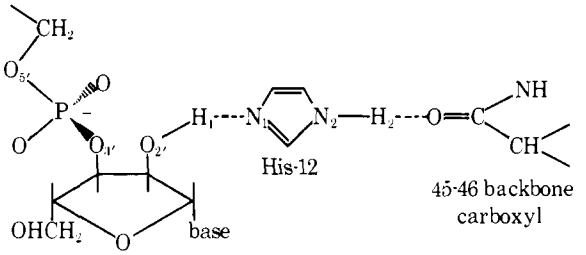
The crystal-structure results[1-4,14,15] indicate that $O_{2'}$ points toward His-12 and may hydrogen bond to it. For this hydrogen bond to be linear, His-12 must rotate 60-80° around the C_1-C_α bond, thereby breaking the hydrogen bond between it and the 45-46 carboxyl backbone.[1,3] It is more likely that His-12 forms a bent, weaker hydrogen bond with $O_{2'}$. The proposed hydrogen-bonded system is depicted schematically below.

The bent hydrogen bond and neutral 45-46 carboxyl

Table XII. Charges per Molecular Unit for Cyclization Step of Transphosphorylation (in Atomic Units)

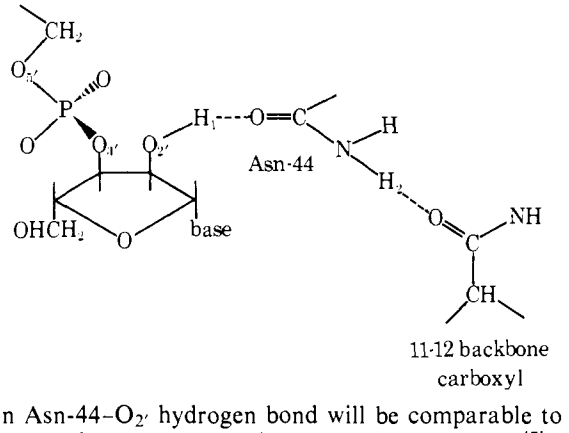
species	Asp-121	His-119	substrate[a]	Lys-41	charge
His-sub		+0.08	−1.08		1−
sub-Lys[b]			−1.31	+0.31	1−
His-sub-Lys[b]		+0.10	−0.47	+0.37	0
Asp-His	−0.12	+0.12			0
Asp-His-sub[c]	−0.53	+0.27	−1.74		2−
Asp-His-sub-Lys[b,c]	−0.50	−0.26	−0.60	+0.36	1−

[a] Substrate is $[PO_5C_2H_7]^{2-}$. [b] Lys-14 is 2.5 Å from the substrate. [c] The proton charge in the hydrogen bond between His-119 and Asp-121 has been divided evenly between them.



backbone make it unlikely that $O_{2'}$ will be totally deprotonated, since the carboxyl backbone is not a good receptor for the positive charge that would develop upon transfer of H_1 to N_1. For this hydrogen-bonded system, when the P–$O_{2'}$ bond is developing, H_1 and H_2 will be approximately midway between the heavy atoms. Nevertheless, these interactions increase the negative charge on $O_{2'}$, making it a better nucleophile and facilitating its addition to the phosphate group.

Recently there has been some doubt raised about whether His-12 actually fulfills the role described above, since there is still significant enzymatic activity when His-12 is blocked[27] or missing.[13] Several possible explanations for this activity have been considered.[13,27a] Another possibility is suggested by the X-ray structure.[1,3] $O_{2'}$ points toward Asn-44 as well as His-12 and may hydrogen bond to either or both groups. The hydrogen-bonded system involving Asn-44 is depicted schematically below. It is clear from the diagram that the $O_{2'}$–Asn-44 and $O_{2'}$–His-12 systems are quite similar.



An Asn-44–$O_{2'}$ hydrogen bond will be comparable to or slightly weaker than a bent His-12–$O_{2'}$ hydrogen bond,[47b] and H_1 will move toward Asn-44 thereby activating $O_{2'}$. Consequently, even though His-12 will be more efficient at activating $O_{2'}$, Asn-44 could conceivably fulfill this role, especially when His-12 is blocked or missing. This suggestion is supported by the following observations: (1) Since H_1 is not totally transferred while the P–$O_{2'}$ bond is developing, all that is required for activation of $O_{2'}$ is the formation of a hydrogen bond. Therefore, "deprotonation" of $O_{2'}$ is essentially independent of the relative basicities of His-12 and Asn-44. (2) Recent experimental work carried out by Gutte[13c] indicates that removal of His-12 does not totally inactivate RNase A analogues, but removal of both His-12 and Asn-44 does.

His-119. His-119 has been postulated to protonate the leaving group $O_{5'}R$, facilitating P–$O_{5'}$ bond breaking when $O_{2'}$ attacks the phosphate group (see Figure 2).[1,4-7,9,48-50] In order to accomplish this, His-119 must rotate with the substrate.[4,51] Crystallographic data show that His-119 has electron density in several positions in the active site and thus could move with the substrate.[1,2,4] Additional X-ray data indicate that the rings of the 5′-base and His-119 interact via base stacking, and this interaction may be responsible for stabilizing His-119 in its catalytic position when the substrate is initially bound.[1] During phosphate rotation the 5′-base is pulled away from its original position[1] and if His-119 follows, the stabilization energy gained from the His-119–5′-base interaction will not be entirely lost, thereby making rotation easier. Furthermore, our calculations for the attachment and partial protonation step show that the hydrogen bond between His-119 and $O_{5'}$ is very strong. Thus, it will take less energy to move His-119 than to break this hydrogen bond. Not only is His-119 then available to protonate the leaving group, but it will also be in a favorable position to accept a proton from a solvent molecule to initiate the hydrolysis stage which follows transphosphorylation.[9,49,50]

Tables VIII (entry 5) and IX (entry 1) demonstrate that the strong hydrogen bond between His-119 and $O_{5'}$ of the substrate results in protonation of $O_{5'}$ even at a large P–$O_{2'}$ distance (2.5 Å). The P–$O_{5'}$ bond is weakened and the P–$O_{2'}$ bond is strengthened almost as much as in the isolated protonated substrate (Table X, entries 2 and 3). The electrophilicity of P and the negative charge on $O_{5'}$ (and $HO_{5'}CH_3$) are decreased correspondingly (Table XI, entries 2 and 3), and the negative charge on the TBP is delocalized (Table XII, entry 1).

Lys-41. There is considerable uncertainty about the distance between the TBP and Lys-41 and its catalytic function.[13b,14-16,53] When the phosphate rotates toward $O_{2'}$, it is believed to rotate toward Lys-41 as well, bringing Lys-41 and the TBP close enough to interact. Lys-41 is proposed to increase the electrophilicity of P and stabilize the TBP by interacting with a phosphoryl oxygen (see Figure 2).[4,7]

Due to the uncertainty in the position of Lys-41, we have made calculations with it placed at both 2.5 and 4 Å from the phosphoryl oxygen. We find that even at a distance of 4 Å, Lys-41 stabilizes the TBP (Table XIII, entry 2). The stabilization originates from the electric field of Lys-41 rather than charge delocalization. When Lys-41 is 2.5 Å from the phosphoryl oxygen, the Lys-41–TBP interaction increases the electrophilicity of P as postulated (Table XI, entries 1 and 4), but it also strengthens the P–$O_{5'}$ and P–$O_{2'}$ bonds (Table X, entries 1 and 4) and delocalizes the negative charge on the TBP (Table XII, entry 2). Our in vacuo calculations exaggerate the interaction, and the reduction produced by the dielectric reaction fields must be kept in mind.

In carrying out our computations, we have assumed that the N–H backbone–phosphoryl oxygen hydrogen bond is no longer present when the phosphate group rotates. If this hydrogen bond is present, the N–H backbone will bring about the same changes in TBP molecular properties brought about by Lys-41, but of smaller magnitude.

Table XIII. Substrate Stabilization for Cyclization Step of Transphosphorylation (in Kilocalories/Mole)

species	ΔE[a]
sub{$[PO_5C_2H_7]^{2-}$}	0
sub-Lys[b]	−117.9
His-sub-Lys[b]	−293.7
Asp-His-sub	−152.1
Asp-His-sub-Lys[b]	−193.8

[a] ΔE = energy of model RNase A active site plus substrate − (energy of RNase A active-site model + energy of isolated substrate), e.g., energy of optimized Asp-His-sub minus energy of optimized Asp-His minus energy of the isolated substrate. [b] Lys-41 is 4.0 Å from the substrate.

His-119-Sub-Lys-41. It is clear from the two previous sections on His-119 and Lys-41 that hydrogen bonds to $O_{5'}$ and to a phosphoryl oxygen produce opposite effects on some of the molecular properties of the TBP. When both are present, compensation between their individual effects occurs and the net result is an increase in the electrophilicity of P (Table XI, entries 1 and 5), a weakening of the P-$O_{5'}$ bond (Table X, entries 1 and 5), a strengthening of the P-$O_{2'}$ bond (Table X, entries 1 and 5), and a large delocalization of the TBP negative charge(Table XII, entry 3). This combination of amino acids produces a strong stabilizing effect on the TBP even with Lys-41 at 4 Å (Table XIII, entry 3). If Lys-41 is close enough to the TBP to form a strong hydrogen bond, the resulting charge delocalization and stabilization of the TBP may be so large that the TBP will not break down even when dielectric polarization effects are properly taken into account. Therefore, we believe the distance between Lys-41 and TBP is greater than the expected hydrogen bond distance of 2.5-2.8 Å. Gutte[13b] also finds that Lys-41 is not essential for catalysis.

Asp-121. The crystal structure of RNase A indicates that Asp-121 is mobile enough to follow His-119 and the substrate when they rotate toward $O_{2'}$.[4] Our conclusion that Asp-121 is not involved electronically in the attachment and partial protonation step leads us to believe that Asp-121 does not rotate with His-119 and the substrate and is not involved in the cyclization step (see Figure 2). This belief is confirmed by the calculations on the Asp-121···His-119···sub and Asp-121···His-119···sub···Lys-41 hydrogen-bonded combinations. Compared to His-119···sub and His-119···sub···Lys-41, the $O_{5'}$-H bond is weaker (Table X, entries 3, 5, 6, and 7) and longer (Table IX, entries 1 and 3) in Asp-121···His-119···sub and Asp-121···His-119···sub···Lys-41 in opposition to what would be expected for its effective participation. Furthermore, Asp-121 causes a large decrease in the negative charge transferred from the TBP, again in conflict with expectations.

Conclusions

For the attachment and partial protonation step of transphosphorylation, we find:

(1) His-119 partially protonates the leaving group ($O_{5'}$R) weakening the P-$O_{5'}$ bond and facilitating an in-line addition of $O_{2'}$.

(2) The 119-120 backbone N-H increases P electrophilicity aiding $O_{2'}$ attack. Gln-11···H_2O has the same function.

(3) The substrate must be hydrogen bonded to His-119 and the N H backbone to activate both P and the leaving group.

(4) A strong His-119-Asp-121 hydrogen bond is unfavorable and is not an important aspect of the catalytic process. The probable function of Asp-121 is to position the adenine ring of the substrate by means of a water-mediated hydrogen bond.

For the deprotonation step of transphosphorylation we find that His-12 is more likely to deprotonate $O_{2'}$, but in its absence Asn-44 may fulfill this role. For the nucleophilic addition step of transphosphorylation we find:

(1) His-119 rotates with the phosphate group and protonates the leaving group facilitating breakdown of the TBP.

(2) Lys-41 increases P electrophilicity and stabilizes the TBP aiding $O_{2'}$ addition.

(3) Lys-41 and the TBP are probably separated by 3 Å or more.

(4) Asp-121 induces unfavorable changes in substrate, does not rotate with the substrate, and is not catalytically involved in this step.

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(26) One possibility suggested in the literature[4] is that His-119 may not be protonated when the substrate is first bound to RNase A. If His-119 is initially neutral and does protonate the leaving group, this suggestion implies that His-119 protonates the leaving group with the hydrogen abstracted from $O_{2'}$. For this step to occur, the abstracted hydrogen must be transferred from the group which deprotonates $O_{2'}$ to His-119. It seems likely from the X-ray structure of RNase A[1,2] that only His-12 could be involved in such a hydrogen transfer. If this is true, it is difficult to account for the enzymatic activity observed when His-12 is blocked[27] or missing.[13] Furthermore, a positive charge on His-119 will aid in stabilizing the negatively charged phosphate group.[4] It also should be noted that a His-12–His-119 proton shuttle is not required for microscopic reversibility.[4] Consequently, we did not consider this possibility when carrying out our calculations.
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Communications to the Editor

The Hydronium Ion (H_3O^+). Preparation and Characterization by High Resolution Oxygen-17 Nuclear Magnetic Resonance[1]

Sir:

First postulated[2] in 1907, the existence of protonated water, the parent oxonium ion, gained wider acceptance with the acid-base theory of Brønsted[3] and Lowry.[4] Hund[5] suggested for H_3O^+ a tetrahedral geometry and Hückel[6] provided the first quantitative discussion of the "anomalous" mobility of the hydrogen ion. The lifetime of H_3O^+ in aqueous solution was estimated to be about ten times longer than the duration of molecular vibrations.[7,8] Experimental work (IR,[9] Raman,[10] ^1H NMR,[11] X-ray,[12] mass spectrometry[13]) reported to date indicates the presence of the hydronium ion, both in solution and solid phase. The strongest evidence for the existence of the long-lived ion was reported by Gold and co-workers.[11e] They observed in the proton spectrum of the partially deuterated HSO_3F-SbF_5-H_2O system in SO_2 or SO_2ClF between −20 and −60 °C three signals, of which one was a triplet assigned to H_2DO^+, another one, an unresolved multiplet, assigned to HD_2O^+, and the third, a singlet, assigned to H_3O^+. However, no direct proof has yet been obtained to unambiguously demonstrate the structure and, particularly, the geometry of this ion.

We present here such a proof, namely the high resolution ^{17}O NMR spectrum obtained from a 1.5 M solution of ^{17}O-enriched water[14] in SO_2, with a slight excess of HF-SbF_5 (1:1 M). At −15 °C a quartet was observed (Figure 1b), which collapsed into a singlet (56-Hz FWHM) upon proton noise decoupling (Figure 1c). The measured ^{17}O–H coupling constant is 106 ± 1.5 Hz and the chemical shift, 9 ± 0.2 ppm downfield from external H_2O (or 496 ppm upfield from SO_2 which was the solvent).

The H_3O^+ spectrum is to be compared with that of H_2O, dissolved in CCl_4 (Figure 1a), where we observed[15] a very sharp triplet (7.6-Hz fwhm, $J_{O\text{-}H}$ = 79.9 Hz) at 12.6 ppm upfield from the broad signal of bulk water (probably present as microscopic droplets in suspension and/or on the wall of the NMR tube).

Our finding clearly demonstrates the tying of three equivalent protons to an oxygen atom in a bona fide covalent species[17] and allows interesting conclusions regarding its properties.

The ion can have a very long lifetime (which implies a significant proton exchange barrier). In fact, convinced that a nonexchanging species would only be observed at very low temperature, we started the experiment at −70 °C (just above the freezing point of SO_2). A very broad oxygen signal was observed, barely revealing a fine structure. This is probably due to hydrogen bonding and/or viscosity effects. Warming the solution resulted in progressive sharpening of the quartet

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