Theoretical Study of a Model for RNA Solvolysis Catalyzed by Large Ribozymes

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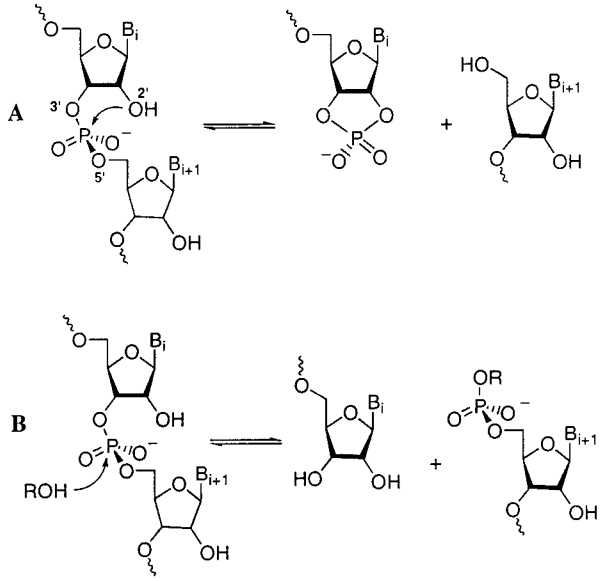
Reaction paths are calculated for a simple model for RNA cleavage catalyzed by large ribozymes. Stationary structures and energies are compared for uncatalyzed solvolysis induced by methanol and methoxide anion in the gas phase with special emphasis on the proposed catalytic effect of internal hydrogen bonding in the ribose moiety at the cleavage site. Other possible effects of solvents and of the ribozyme on the reaction mechanism are briefly discussed. It is proposed that the primary step of the catalyzed reaction is deprotonation of the external nucleophile and that the rate of the reaction is determined by the final cleavage step.

Introduction

Splicing of mRNA is a mechanism to remove noncoding DNA sequences (introns) from RNA copies. This process is autocatalyzed by the introns themselves, known as large ribozymes, with or without help from other RNA molecules and/or proteins, and requires the assistance of bivalent metal ions, such as Mg^{2+}.[1] The corresponding transesterefication starts with an attack of an exogenous nucleophile, either a hydroxyl group or its anion, on the phosphodiester group and leads to cleavage at the 3′-OP site of the ribose moiety. This mechanism differs from the "internal" mechanism of RNA cleavage encountered in normal acid/base catalysis and catalysis by small ribozymes, in which the adjacent 2′-OH group attacks the phosphate, which leads to cleavage at the 5′-OP site. With a few recently discovered exceptions, the mechanism catalyzed by small ribozymes also requires assistance of bivalent metal ions, one of whose functions may be to strip the 2′-OH group of its proton prior to the attack. For recent reviews of the mechanisms of RNA cleavage mediated by small and large ribozymes we refer to articles by Pyle,[2] Kuimelis and McLaughlin,[3] and Takagi et al.[4] The basic processes are illustrated in Scheme 1. Scheme 1A shows that the internal attack by 2′-OH proceeds through a sterically favorable intermediate five-membered ring structure. To make the external mechanism of Scheme 1B competitive, it will be necessary for the exogenous nucleophile to be present at the right place, in the right form, and with the right orientation. In addition it may be desirable for the 2′-OH group to be deactivated or reoriented. All of this presumably is accomplished by large ribozymes, which appear in a number of different forms. On the basis of their structure and mode of operation, they are divided into three groups, group I and group II introns and the ribonuclease P ribozyme, but the detailed mechanism of their catalytic action is not well understood at present.

In a recent experimental investigation of the transesterefication reaction catalyzed by the *Tetrahymena* ribozyme, Herschlag, Piccirilli, and their co-workers,[5] addressed the part played by the 2′-OH group in the cleavage process. This large ribozyme, derived from a self-splicing group I intron, catalyzes a reaction in which an exogenous guanosine nucleophile cleaves a specific phosphodiester bond. Through a series of substitutions at the 2′-OH and neighboring sites, the authors provided direct evidence that the cleavage is assisted by formation of a hydrogen bond of the 2′-OH group with the adjacent bridging 3′-oxygen rather than with the nonbridging oxygens, in line with what had been generally speculated. No evidence was found for a direct catalytic effect of divalent metal ions such as Mg^{2+} and Mn^{2+} on the activity of the 2′-OH group, although these ions have a strong effect on the 3′-OP bridge and the exogenous 3′-OH nucleophile of guanosine.[6] From the observed 1000-fold decrease in the cleavage rate upon substituting 2′-H for 2′-OH, it was concluded that the 2′-OH- -3′-O hydrogen bond stabilizes the transition state by about 4 kcal/mol. This conclusion is surprising since in aqueous solution the pK_a values of the 3′-OH groups in ribose and 2′-deoxyribose are very similar, namely 12.1 and 12.6, respectively.[7] To account for this apparent

SCHEME 1



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contradiction, the authors[5] suggest that the 2′-OH group is part of a hydrogen-bonded network that controls its behavior, as proposed earlier by Strobel and Ortoleva-Donnelly.[8]

In another recent experimental investigation, a group led by one of us[9] addressed these problems by studying the cleavage reaction in an organic solvent (CH_2Cl_2:MeOH 70:30) rather than in water. The system studied was not the ribozyme itself but a simplified RNA model in which the uncatalyzed reaction proceeds quickly enough to be monitored by standard methods. It was observed that under these conditions cleavage initiated by an exogenous methoxide anion, present in concentrations of up to 0.6 mol of NaOMe/L, is competitive with internal cleavage. It was suggested that hydrogen bonding of the 2′-OH group to the bridging 3′-oxygen is a contributing factor, since such an effect has been recognized in acyl transfer reactions, where it is referred to as the Henbest-Kupcham effect.[10]

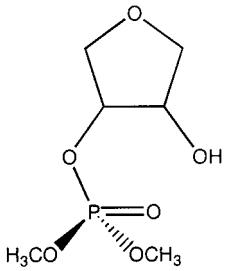
In view of these observations, it seems timely to apply theoretical methods to the analysis of the cleavage reaction catalyzed by large ribozymes. Quantum-chemical studies of the alternative internal cleavage catalyzed by small ribozymes[11] have contributed significantly to our understanding of its reaction mechanism. There have been few theoretical studies, however, of the external cleavage process. It is obviously more complicated due to the presence of the exogenous nucleophile and to the presumed necessity of controlling the nucleophilicity of the 2′-OH group. Given this situation, it seems prudent to start with a simple system and to add complexity in a stepwise fashion. In particular we need to address the uncatalyzed reaction, as it forms the basis on which to build our understanding of the catalytic process. Therefore we first consider a model similar to the uncatalyzed reaction studied by Roussev et al.[9a] In this experiment exogenous cleavage was achieved by reducing the polarity of the solvent. Practical limits were set by the need to dissolve the nucleotide and the NaOMe nucleophile. In theoretical calculations, where there are no such limits, we can minimize the polarity by eliminating the solvent altogether. Therefore we probe the mechanism of the cleavage reaction in the gas phase, which will exaggerate the effect of reducing the solvent polarity and thus may serve as a useful test of the proposed mechanism. A simple way of extending the calculations to a polar medium is the PCM model,[12] in which the system is enclosed in a cavity inside a dielectric continuum. This should be an acceptable first-order approximation for organic solvents that form no hydrogen bonds with the substrate, but may not yield accurate results when protic solvents,[13] polar groups of the intron,[8] and associated metal ions[6] are present.

The exogenous nucleophile in the reaction catalyzed by group I introns is the 3′-OH group of the guanosine cofactor; for group II introns it is usually the 2′-OH group of an adenosine that is part of the intron sequence.[1−4] In both cases and also for the RNAse P ribozyme, it may simply be a water molecule. In our model calculations we use a methanol molecule. Proper initial placement of this molecule allows the cleavage to proceed via the external mechanism and should provide information about the role of the 2′-OH group in this process. Guided by the results of the gas-phase calculation, we may then be able to infer whether specific interaction at the 2′-OH site are needed to reproduce the proposed catalytic effect of this group on the cleavage reaction.

Models and Computational Procedures

To study the biomimetic reaction mechanism of Scheme 1B, we use a simplified model of RNA solvolysis in *Tetrahymena*. We replace the uridine base preceding the scissile 3′-phosphate

CHART 1



by a 3,4-dihydroxytetrahydrofuran 4-dimethyl moiety (see Chart 1), the leaving 5′ adenosine phosphate by methyl phosphate, and the attacking guanosine 3′-OH (or adenosine 2′-OH or water in other large ribozymes) by a methanol molecule. This nucleophile is placed in a position such that it will attack the phosphate moiety from a direction opposite to the scissile bond. Presumably this mimics part of the catalytic action of the large ribozyme. To model the model reaction in organic solvents, we use the analogous 3,4-dihydroxytetrahydrofuran 4-dimethyl phosphate as the nucleotide and the methoxide anion as the nucleophile.[9]

To evaluate the potential-energy surface of the reaction, we optimized the stationary structures appearing along the reaction path, using the GAUSSIAN 98 suite of programs,[14] and verified all optimizations by vibrational analysis. The results obtained at the Hartree−Fock (HF)/6-31+G* level proved unsatisfactory in that the energies were very sensitive to correlation effects when tested by single point second-order Møller−Plesset (MP2) calculations.[15] Since full optimization at the MP2 level is computationally very demanding for the present system, we switched to density functional (DFT) calculations with a B3LYP functional[16] and a 6-31+G* basis set. The corresponding relative energies were generally close to those obtained at the MP2/6-31+G*//HF/6-31+G* level. To check the adequacy of the basis set, a few single point calculations were carried out with the 6-311++G** basis set, which were found to produce comparable results. In addition, potential-energy profiles corrected for zero-point energy differences (ΔZPE) as well as Gibbs thermal energy profiles (ΔG) were evaluated.

Solvolysis by a Methoxide Anion

First we consider the experiment by Roussev et al.,[9a] in which the model nucleotide is cleaved following attack by a methoxide anion on the phosphate in an organic solvent. This reaction is not observed in aqueous solution, which indicates that the relatively low polarity of the medium redirects the reaction so as to favor external attack. This effect should be even stronger in our model calculations for the gas phase. The calculated stationary structures along the reaction path are illustrated in Figure 1, which also indicates the interatomic distances pertinent to the reaction. An initial ion−dipole complex (**C1**) is formed between the model nucleotide and the methoxide anion. The anion then attacks the phosphate triester to form a pentacoordinated, trigonal-bipyramidal intermediate (**Int**) via a pentacoordinated transition state (**TS1**).[17] This is the first step of the reaction. It is followed by a second step in which the 1,2-diolate anion **Int**, formed in the first step, is cleaved to form the final complex (**C2**) via a second pentacoordinated transition state (**TS2**). The Gibbs free energy profile along the reaction path calculated at the DFT/B3LYP/6-31+G* level is illustrated in Figure 2; the corresponding energies are listed in Table 1. Similar results (not shown) are obtained at the MP2/6-31+G*//HF/6-31+G* level.

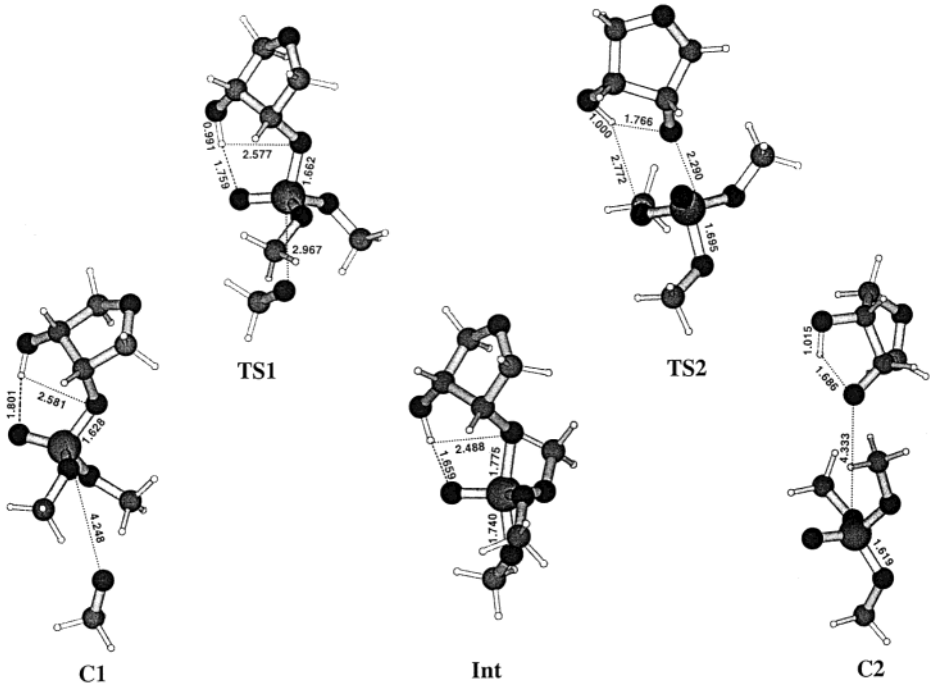


Figure 1. Transesterefication mechanism of 3,4-dihydroxytetrahydrofuran 4-dimethyl phosphate by methoxide, calculated at the B3LYP/6-31+G* level. The dark spheres represent oxygen atoms and the dotted lines depict hydrogen bonds.

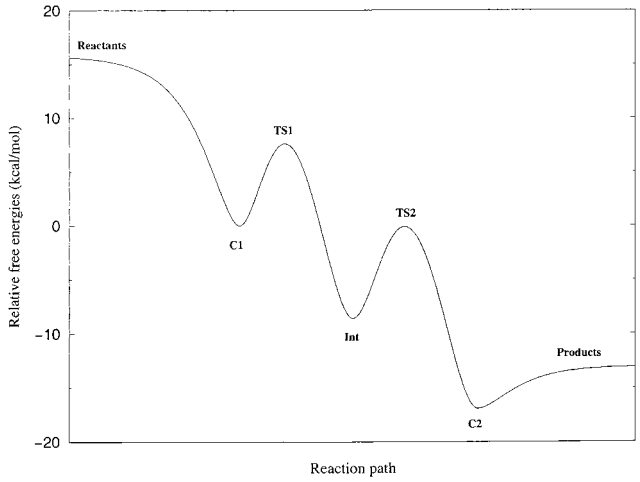


Figure 2. Gibbs free energy profiles calculated at the B3LYP/6-31+G* level for the reaction depicted in Figure 1.

Because of the complete absence of solvent in these calculations, the results cannot be expected to give an accurate representation of the reaction in the organic solvent, whose dielectric constant is estimated to be about 16. To mimic the effect of the solvent on the relative stability of **Int** and **TS2**, we have used the PCM model[12] for dichloromethane, which has a dielectric constant of 9. We found that at this level the effect of the dielectric continuum on the relative energies is negligible. However, this approach may be unreliable, since the solvent mixture used contains methanol, so that there may also be local hydrogen-bonding effects, which are not reproduced by a continuum model. Without a specific investigation of such effects, which will be computationally very demanding, the calculations cannot be used directly to establish which of the two steps of the cleavage reaction is rate-determining. However, indirect arguments to be presented below suggest that is the cleavage step.

The calculations allow a clear prediction about the role of the 2′-OH group, the elucidation of which is our main purpose.

TABLE 1: Relative Energies (kcal/mol) [Electronic (ΔE), after ZPE Correction ($\Delta E + \Delta ZPE$), and Gibbs Free Energies at Room Temperature (ΔG)] along the Transesterification Reaction Path for Methoxide Anion Attack on the Ribozyme Model Calculated at the B3LYP/6-31+G* Level

species	ΔE	$\Delta E + \Delta$ZPE	ΔG
reactants	25.6	24.6	15.8
C1	(0)[a]	(0)[b]	(0)[c]
TS1	4.9	5.2	7.6
Int	−14.4	−12.1	−8.6
TS2	−3.4	−2.2	−0.1
C2	−16.7	−15.6	−16.9
products	−2.2	−2.4	−13.0

[a] Absolute energy = −1114.38955 au. [b] Absolute energy = −1114.14337 au. [c] Absolute energy = −1114.19375 au.

From the bond length indicated in Figure 1 it follows that there is no significant difference between 2′-OH- -3′-O hydrogen bonding in **C1** and **TS1**, which indicates that the catalytic effect of the 2′-OH group on the first step of the reaction is negligible. In both structures 2′-OH is a moderately strong hydrogen-bond donor to a nonbridging oxygen and a very weak donor to the bridging oxygen. It equally follows from the data in Figure 1 that in the second step the strength of the hydrogen bond with the nonbridging oxygen decreases from strong to negligible between **Int** and **TS2**, while that with the bridging oxygen increases from very weak to moderately strong. Thus, overall, the strength of this hydrogen bonding does not increase and hence the strong hydrogen bond in **C2** cannot have a significant catalytic effect on the second step of the reaction. It appears, therefore, that whichever step is rate-determining, a nonpolar medium does not favor a catalytic contribution of the 2′-OH group to the rate of cleavage. This leaves open the possibility of hydrogen bond donation of the methanol component of the solvent mixture to the 2′-OH site, which might in turn lead to stronger 2′-OH- -3′-O hydrogen bonding. The calculations suggest that if there is external hydrogen-bond donation to the nonbridging oxygen, it is more likely to occur in the second step of the reaction.

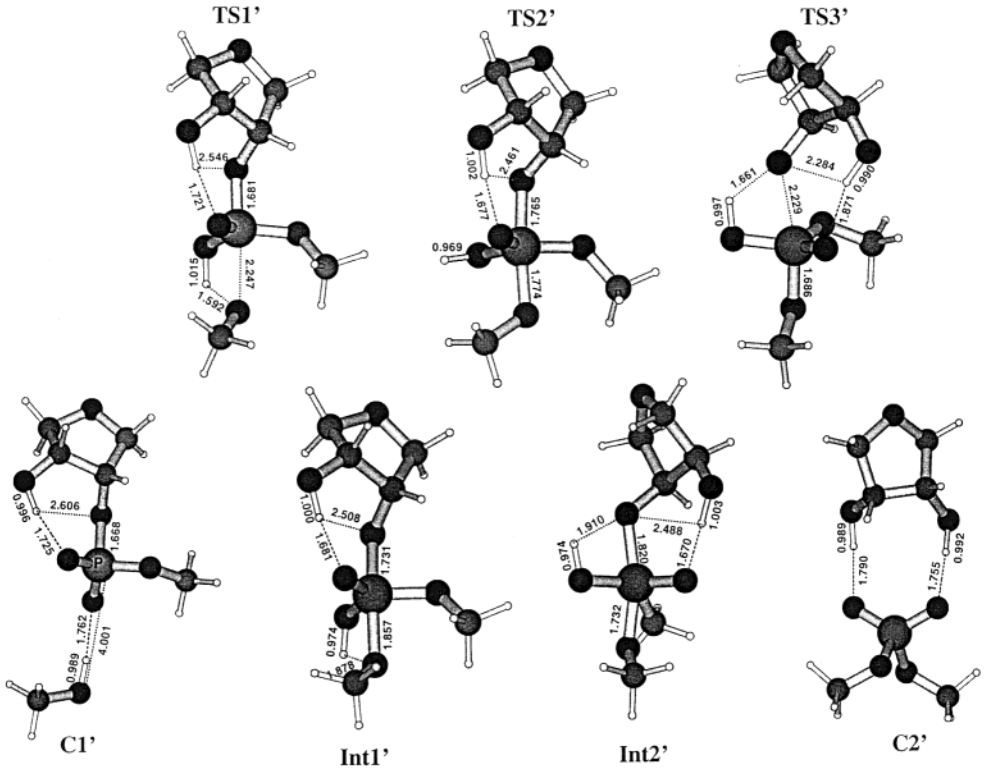


Figure 3. Transesterification mechanisms of 3,4-dihydroxytetrahydrofuran 4-dimethyl hydrophosphate by methanol, calculated at the B3LYP/6-31+G* level. The dark spheres represent oxygen atoms and the dotted lines depict hydrogen bonds.

TABLE 2: Same as Table 1, along the Solvolysis Reaction Path for Methanol Attack

species	ΔE	$\Delta E + \Delta ZPE$	ΔG
reactants	14.7	13.5	5.9
C1′	(0)[a]	(0)[b]	(0)[c]
TS1′	36.1	35.2	39.4
Int1′	33.4	33.7	38.0
TS2′	42.9	42.0	46.9
Int2′	30.5	31.0	35.5
TS3′	35.3	34.7	38.5
C2′	−2.1	−1.6	0.3
products	25.4	24.2	15.1

[a] Absolute energy = −1105.16885 au. [b] Absolute energy = −1104.94773 au. [c] Absolute energy = −1104.99825 au.

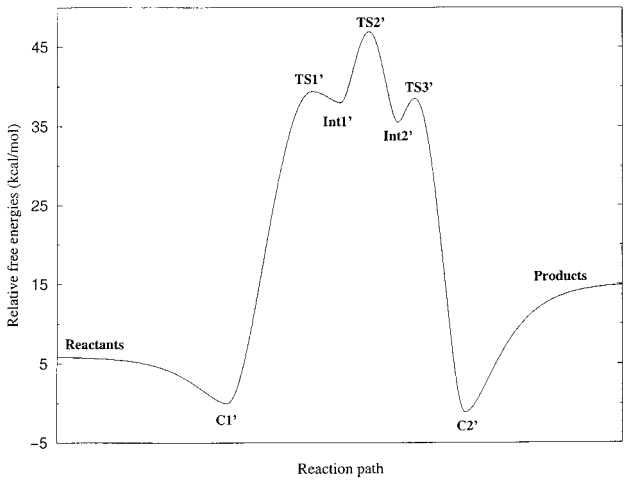


Figure 4. Gibbs free energy profiles calculated at the B3LYP/6-31+G* level for the reaction depicted in Figure 3.

Solvolysis by a Methanol Molecule

In the experiment of Yoshida et al.,[5] the attacking nucleophile is the 3′-OH group of guanosine; in our calculations we represent it by a methanol molecule. The calculated stationary structures along the reaction path of our model system are illustrated in Figure 3, which also presents a selection of pertinent interatomic distances. The corresponding Gibbs free energies, listed in Table 2, are depicted in Figure 4. The initially formed complex (**C1′**) has a moderately strong hydrogen bond with a nonbridging oxygen. This complex forms an anionic intermediate state (**Int1′**), in which the proton is fully transferred along the hydrogen bond to the nonbridging oxygen while maintaining a strong hydrogen bond with the methoxy oxygen. Simultaneously, the methoxy group is attached to the phosphorus to form a trigonal-bipyramidal structure. This is a complex, highly endothermic reaction that is unlikely to proceed without catalysis. The two new bonds are formed in a concerted way but not synchronously. The proton transfer is virtually complete in the transition state (**TS1′**), while the new axial P−O bond is still being formed, the P−O distance of about 225 pm being intermediate between the distance in **Int1′** (about 186 pm) and that in **C1′** (about 400 pm). As expected for a highly endothermic reaction, **TS1′** approaches **Int1′** both in structure and energy. In particular, as follows from the bond lengths in Figure 3, the hydrogen bond of the 2′-OH group with the free phosphate oxygen is slightly stronger in **Int1′**, but that with the bridging oxygen remains very weak.

This first step in the cleavage reaction is followed by a second step in which the P−O−H proton moves from the internal phosphate hydrogen bond that points toward the methoxy oxygen to a hydrogen bond with the bridging oxygen. Explicit calculations show that for this step pseudorotation[18] is energetically unfavorable compared to internal rotation of the (P)OH group via the transition state **TS2′**. Apart from this proton displacement, which is likely to proceed via rapid quantum-mechanical tunneling through the relatively low barrier, the structures of **Int1′** and **Int2′** are similar. The phosphorane

fragments have a trigonal-bipyramidal structure and the hydrogen bonding involving the 2′-OH group remains the same. In the third, highly exothermic step of the reaction, cleavage takes place, the proton being transferred to the leaving vicinal diol, which is reached via a low-energy transition state (**TS3′**) that is very similar to **Int2′** with a very weak 2′-OH- -3′-O hydrogen bond. The final complex (**C2′**) has a loose structure held together by a moderately strong hydrogen bond; there is no significant internal hydrogen bond in the diol.

The calculations indicate that the first step in the reaction is rate-determining in sharp contrast to what has previously been found[11] for the internal cleavage mechanism catalyzed by small ribozymes, where the final, cleavage step determines the rate. The greater effort needed to complete the first step of the external compared to the internal reaction in the absence of catalysis by ribozymes is in accord with the observation that the external process requires a catalyst of much greater complexity. The catalyzed mechanism may therefore differ in many respects from the uncatalyzed reaction studied here. Specifically, the calculated high activation energy and endothermicity and the observed strong catalytic effect for divalent metal ions associated with the guanosine nucleophile[5,6,19] suggest a preliminary step in which the attacking OH group loses a proton, possibly to a hydroxide ion associated with the metal ion. This would bring the mechanism closer to that calculated for the methoxide attack, for which we found the activation energy and endothermicity of phosphorane formation to be much smaller. It would also eliminate the need for a proton-transfer step to reorient the proton toward the bridging 3′-O (the second step of the preceding section). In that case the final, cleavage step may become rate determining, as found for the reaction catalyzed by small ribozymes.[11] However, in the present calculations, the corresponding final transition state **TS3′** in Figure 3 shows, just as the transition state of the first step **TS1′**, no indication of significant 2′-OH- -3′-O hydrogen bonding, contrary to the experimental evidence presented by Yoshida et al.[5] The same was observed in the calculations of the preceding section on the model reaction induced by a methoxide anion. As we argued there, we expect that introduction of solvent in the form of a polarizable continuum will not remove this discrepancy, although specific local solvent interactions may conceivably be effective. In the catalyzed reaction it is likely that the large ribozyme will provide the necessary interaction at the 2′-OH site.[5,8]

Discussion

The calculations indicate that the first step in the uncatalyzed attack by an exogenous hydroxyl group on the phosphate moiety of RNA is a concerted reaction in which abstraction of the proton by a nonbridging oxygen is followed by addition of the methoxy group to the anionic phosphate moiety. This process is highly endothermic in the gas phase and yields a structure with the transferred proton pointing in the wrong direction for cleavage, thus requiring an additional (P)OH internal rotation, after which cleavage is an easy process. The overall reaction can be accelerated by the formation of a 2′-OH- -3′-O hydrogen bond in the transition state of the rate-determining step. Because such a contribution is not found for the uncatalyzed reaction, it must be a catalytic effect produced by the large ribozyme. This agrees with the conclusion reached by Strobel and Ortoleva-Donnelly,[8] and Yoshida et al.[5] These authors noted that the catalytic activity of the 2′-OH group in *Tetrahymena* is connected to its participation in a hydrogen-bonding network, in which it acts as the acceptor of a hydrogen bond donated by A270. However, the estimated energy gain of forming the 2′-OH- -3′-O hydrogen bond is only about 10% of the calculated barrier height, which is not nearly enough to reduce it to a value compatible with the observed rate of reaction.[3] We therefore suggest that the major effect of the catalysis, apart from linking the exogenous nucleophile to the reaction site, is deprotonation of the attacking hydroxyl group. Our calculations on the model system of ref 9 indicate that 2′-OH- -3′-O hydrogen bonding is not a secondary effect of such an anionic attack but, if real, is induced separately by the ribozyme, thus illustrating its ability to affect two or more sites simultaneously.

The logical next step in this study is to extend the model by the introduction of an external methanol molecule that mimics A270 by donating a hydrogen bond to the 2′-OH group. Preliminary calculations of this type have been carried out and show indeed that this leads to stronger hydrogen bonding of 2′-OH with nonbridging oxygens. The complexity of this extended model has thus far prevented a more definite analysis of the effect of this external methanol on 2′-OH hydrogen bonding with the bridging oxygen.

In summary, on the basis of these calculations we offer the following suggestions. (i) The external nucleophile is likely to be deprotonated with the assistance of bivalent metal ions before it attacks the phosphate. (ii) The observed catalytic effect of 2′-OH- -3′-O hydrogen bonding requires ribozyme assistance, possibly at the 2′-OH site by hydrogen bonding and the 3′-O site by divalent metal ions. Since (ii) is most likely to occur in the final, cleavage step, we further suggest that this step is rate-determining in the catalyzed reaction.

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