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Metal ions and the stereochemistry of ribozyme reactions

Ralf Stowasser [1] and David A. Usher *

Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853-1301, USA

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Abstract

Inversion of configuration at phosphorus during ribozyme-catalyzed cleavage of RNA is usually considered unequivocal proof of in-line attack, but the relevant pseudorotation diagram for formation of the 2′,3′-cyclic phosphate shows that inversion is not inconsistent with adjacent attack as long as breakdown of the trigonal bipyramid is in-line. For the reaction to occur by adjacent attack, a normally unstable apical oxyanion in the trigonal bipyramidal intermediate would have to be stabilized. Density-functional calculations show that a metal ion such as magnesium could perform this stabilization. We conclude that the possibility of adjacent attack should not be too hastily dismissed in cases where the setup is closer to adjacent than to in-line geometry.

Keywords: Adjacent; In-line; Trigonal bipyramid; Magnesium; Phosphate; Pseudorotation; Hammerhead

* Corresponding author. Fax: +607-255-4137.
E-mail address: daul@cornell.edu (D.A. Usher).
[1] Present address: BAYER AG, Patent Department Q18, 51373 Leverkusen, Germany.

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1. Introduction

During the 1960s the study of a large number of reactions involving nucleophilic attack on pentavalent phosphorus led to the formulation of a set of "preference rules" [1]. One biochemically important reaction of this type is nucleophilic attack on phosphorus by a hydroxyl group, a fundamental reaction in the formation and cleavage of RNA in the cell. According to the preference rules, bimolecular nucleophilic displacements in phosphorus take place by way of a trigonal bipyramidal intermediate or transition state (TBP), and the incoming nucleophile occupies an apical position in the initial TBP. Similarly, as the leaving group is about to depart, it does so from an apical position. Other relevant rules are that a five-membered ring that includes phosphorus spans one basal and one apical position in the TBP, and relatively electronegative groups prefer apical positions while electropositive groups prefer basal positions. A phosphate oxyanion is relatively electropositive compared to a P–OH or P–OR.

Consideration of these rules led one of us [2] to introduce the terms "in-line" and "adjacent" to describe two possible geometries for the reaction that is catalyzed by the enzyme ribonuclease-A, and these terms have since come into more general use to describe the geometry of nucleophilic displacement reactions of phosphate esters. If the nucleophile, the phosphorus and the leaving group are all in a straight line (Fig. 1a), this is an in-line mechanism. In the alternative adjacent mechanism (Fig. 1b), the nucleophile again adds in an apical position, but the eventual leaving group initially occupies a basal position, and before departing must be placed in an apical position, for example, by the process of pseudorotation [3]. In the case of adjacent attack on an internucleotide phosphate diester by a 2′-hydroxyl group, a phosphorane oxyanion would be forced to take up an apical position in the first-formed TBP, and this is considered energetically unfavorable. In 1970, in the first application of

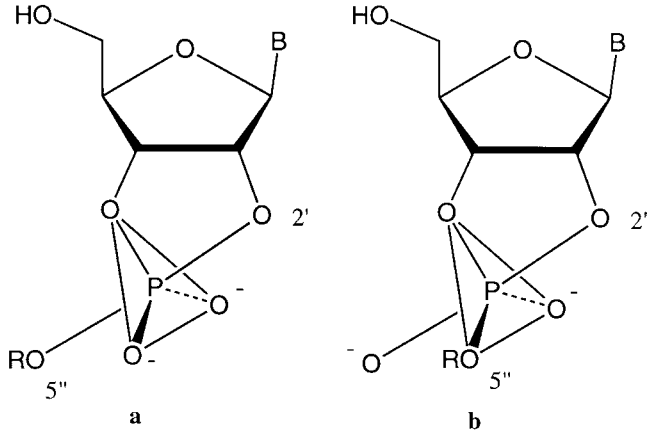

Fig. 1. The structure of the initial trigonal bipyramid formed during breakage of RNA by intramolecular attack of the neighboring hydroxyl group: (a) in-line attack, (b) adjacent attack. In both cases the nucleophile is the 2′-O and the leaving group is the 5″-OR.

these concepts to an enzyme-catalyzed reaction, we proved an in-line mechanism for the action of RNase-A on a RNA substrate [4], and subsequent investigations of this and other enzyme-catalyzed reactions came to similar conclusions [5–8]. These ideas also allowed prediction [9] and experimental verification [10] of the relative reactivity of 2′,5′ and 3′,5′ internucleotide bonds towards non-enzymic cleavage. More recently, an "in-line fitness" parameter has been devised that predicts the approximate ease of cleavage at any given internucleotide linkage, based on its geometry [11].

The method that we used to determine the geometry of the two steps of the ribonuclease-A reaction was to check if there was inversion of configuration at the phosphorus that was undergoing attack, and this type of test has been widely adopted. Following the lead of two influential reviews of the early 1980s [7,8], inversion at phosphorus invariably has been taken to mean that an in-line displacement has occurred. What has not been sufficiently recognized, however, is that for cleavage of RNA by formation of the 2′,3′-cyclic phosphate, the stereochemical finding of inversion does not prove that in-line *attack* by the 2′-hydroxyl has occurred. To the extent that classical pseudorotation [1] applies to these reactions, what one may infer from inversion is that in-line *breakdown* has occurred; i.e., just as the TBP is about to break down to cyclic phosphate, the 5′-oxygen leaving group and the original 2′-oxygen nucleophile must be in-line with the phosphorus. Inversion does *not* reveal the relative geometry of nucleophile and leaving group in the first-formed trigonal bipyramid, and adjacent attack does not necessarily lead to retention of configuration. This can be seen from the pseudorotation diagram (Fig. 2). Thus we could start with the adjacent setup of structure *Y*, follow the path [2′,O], [3′,S], [2′,OR], and get to the same exo-*S* cyclic phosphate that we would have got to directly if we had started with the in-line setup of structure *X*. (Structures *X*, *Y*, and *Z* in Fig. 2 are merely different conformations of the same molecule. They interconvert by rotation about the P–3′O bond, and all three have the same configuration at phosphorus). The relationships of Fig. 2 apply not only to the first step of RNase-A action [12], but also to those ribozymes that cleave RNA by attacking phosphorus with the 2′-OH to displace the 5′-OH and give a 2′,3′-cyclic phosphate diester. For the first step of RNase-A action the stereochemical evidence [5] proves an in-line geometry for breakdown of the pentaoxyphosphorane, but by itself does not rule out adjacent attack. In the second step of RNase-A action (ring-opening of the 2′,3′-cyclic phosphate), attack is unambiguously in-line [4], but breakdown may not be.

However, if enzyme- or ribozyme-catalyzed cleavage of RNA were to involve adjacent attack, then something would have to stabilize a normally unstable apical P–O^- (e.g., that in structure [2′,O] in Fig. 2). One reasonable candidate for this role is a metal cation. To see if this sort of stabilization could occur, we have performed a set of detailed calculations, the results of which show that when magnesium is present, adjacent attack is indeed a possibility for this type of reaction. According to Zhou and Taira [13], a monoanionic TBP may have a long enough life to be called an intermediate, whereas a dianionic TBP does not. This observation is consistent with a cationic species binding to an apical P–O^- and thereby stabilizing the TBP and allowing time for pseudorotation to occur. Ribonuclease-A has no metal ion requirement, and it is likely that both attack and breakdown are in-line, for both steps.

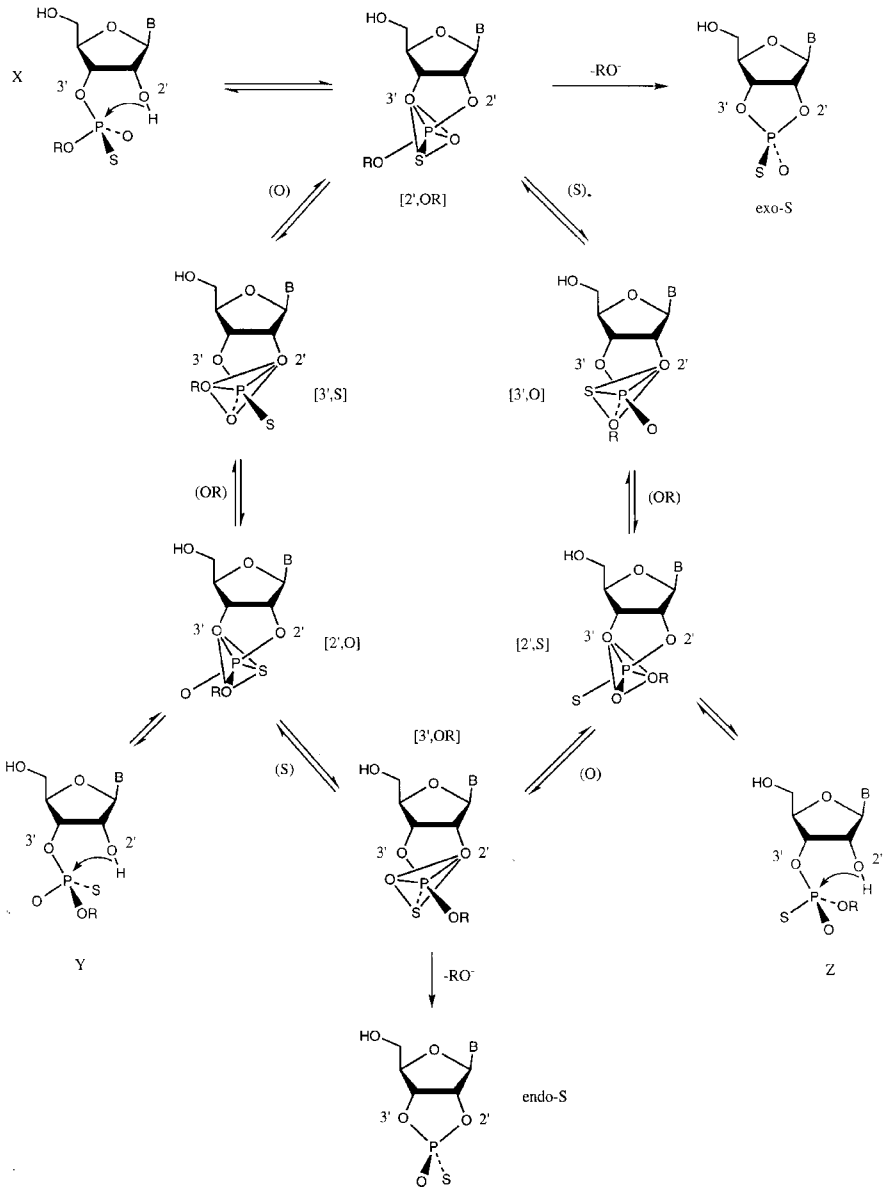

Fig. 2. A pseudorotation diagram showing the formation, interconversions, and decomposition of a cyclic phosphorane intermediate. Charges and double bonds have been omitted for clarity. All three acyclic diester structures (*X*, *Y*, and *Z*) are the same S_P phosphorothioate.

2. Computations

We modeled the reactive intermediate or transition state by structure **1** (Fig. 3), in which we substituted the sugar-unit by a C_2H_4 bridge and replaced the 5′-OR group

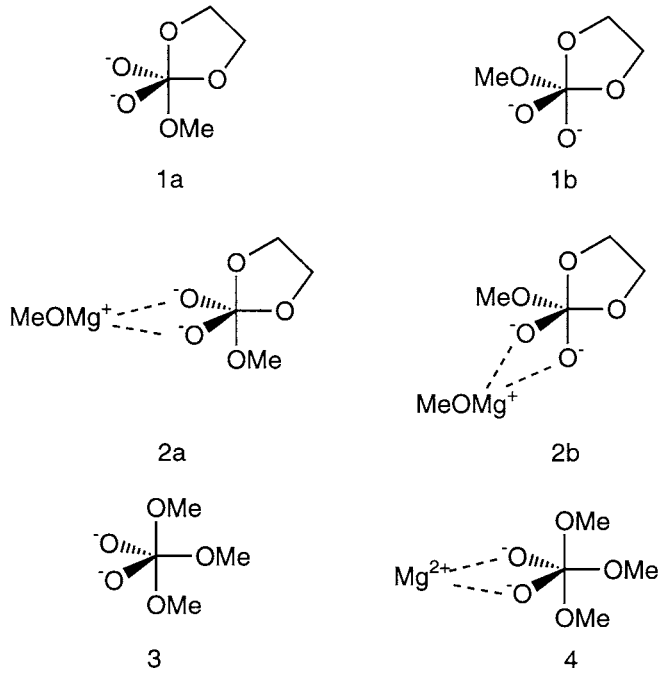

Fig. 3. Pentaoxyphosphorane models used in this and other [18] studies. Structures **1a** and **2a** resemble intermediate [2′,OR] in Fig. 2, while **1b** and **2b** correspond to structure [2′,O]. The phosphorus atom at the center of each trigonal bipyramid is not shown.

by an OMe group. The resulting simplified molecule is small enough that we could investigate possible magnesium cation stabilization of **1a** and **1b** with high level density-functional calculations. Input geometries were generated by the application of molecular mechanics (MM2), implemented within the SPARTAN 3.0 program [14]. A full optimization of the geometry with the high quality relatively large triple-zeta basis set used in the BP86 DF calculation is extremely computer time consuming. To save computer time, we pre-optimized the geometries with the semi-empirical PM3 method within the program SPARTAN 3.0. Using these pre-optimized structures we obtained convergence of the optimization procedure often after only a few cycles. Semi-empirical self-consistent field (SCF) calculations were performed with the PM3 method (program SPARTAN 3.0 [14]). Density-functional calculations were performed with the Amsterdam density functional (ADF) program package, using a Becke Perdew (BP86) density functional and the flexible triple-zeta basis set type V [15,16]. All structures were optimized at the density-functional level without geometrical constraints or symmetry restrictions. Vibrational frequency calculations were applied to characterize the calculated geometries as minima or transition structures. Characterization of the density-functional optimized structures was done by a BP86/DZVP method, and verified by HF/3-21G calculations on HF/3-21G geometries. For estimation of solvent (water) effects the SCF-IPCM method, implemented in the Gaussian 94 program package [17] was applied.

Uchimaru et al. [18] performed detailed calculations on model **3**, which is similar to our model **1**, but which has two methyl groups instead of the C_2H_4 bridge. They showed with Hartree–Fock calculations that **3** is a stable stationary point on the energy hypersurface with one imaginary eigenfrequency. This was assigned to rotation of the apical methoxy groups. We obtained the same results on our model **1a** by standard semiempirical PM3 calculations. Energy differences resulting from different conformations of the apical methoxy group of **1a** are not significant in this discussion [18]. It is clear from our calculations that **1b**, which can be generated from **1a** by a pseudorotation, is not stable, and indeed, optimization of **1b** led back to **1a**. In contrast to the stability of structure **3**, Uchimaru et al. [18] were unable to find a stable fully optimized stationary point for the Mg^{2+} complexed phosphorane derivative **4**. However, they found under the constraints of the internal coordinates of the dianionic oxyphosphorane a movement of the Mg^{2+}, which they initially placed between the two basal negative oxygens, but towards one of the apical oxygens of **4**. Our reinvestigation of this problem on model **2a** with density-functional methods (full optimization; extensive transition structure as well as minimum search) led to the same result: a stationary point for this pentacoordinated species could not be located.

However, we found that for the Mg^{2+} or $MeOMg^{+}$ complexed isomer **2b**, which has one basal and one apical P–O^{-}, there is a stationary point on the energy hypersurface with one imaginary normal-mode frequency (47 cm^{-1}), unambiguously assignable to the rotation of the basal methoxy group. This is the type of intermediate that would be generated by adjacent attack on phosphorus. (We tested these calculations on $MeOMg^{+}$ as well as on Mg^{2+} since the magnesium in a ribozyme is unlikely to exist as the free dication. The results were the same). The geometry of this structure is shown in Fig. 4. This type of structure, with one basal and one apical P–O^{-}, apparently was not investigated by Uchimaru et al.

Our calculations show that a magnesium ion destabilizes structure **2a**, and stabilizes the otherwise unstable stereoisomer **2b**. This density-functional result was additionally verified by Hartree–Fock RHF/3-21G calculations. Although the role of solvent is not clear in the case of ribozyme reactions, an estimation of the solvent (water) effect by single point self-consistent reaction field-polarized continuum model (SCRF-IPCM) calculations on DF geometry **2b** and the hypothetical structure **2a** also favors structure **2b** by about 58 kJ/mol.

3. Ribozymes

The reaction of the hammerhead ribozyme had been shown to proceed with inversion of configuration at phosphorus [19–21]. Thus when two independent X-ray crystal structures were solved [22,23] it was considered somewhat surprising that both showed a setup closer to that expected for adjacent rather than in-line attack (Fig. 5). This situation has been commented on at some length [24]. Two previously published systems that also appeared setup for adjacent attack are a tRNA–lead complex [25], and a RNA–manganese complex [26]. All three of these systems were believed to require the presence of a divalent metal ion for activity; in the case of the

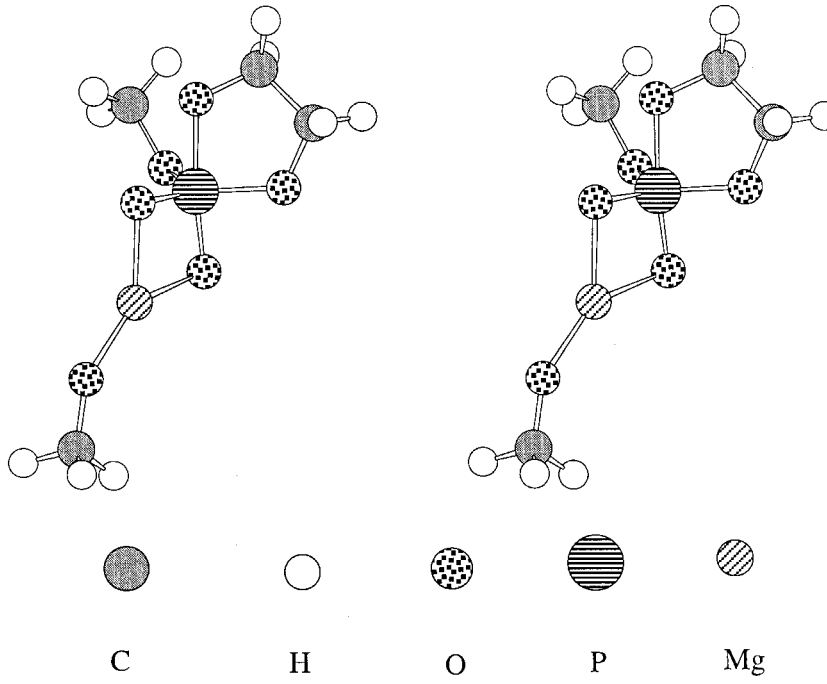

Fig. 4. A stereoview of the optimized structure of the stabilized complex of $MeOMg^{+}$ with a dianionic pentaoxyphosphorane (**2b** in Fig. 3).

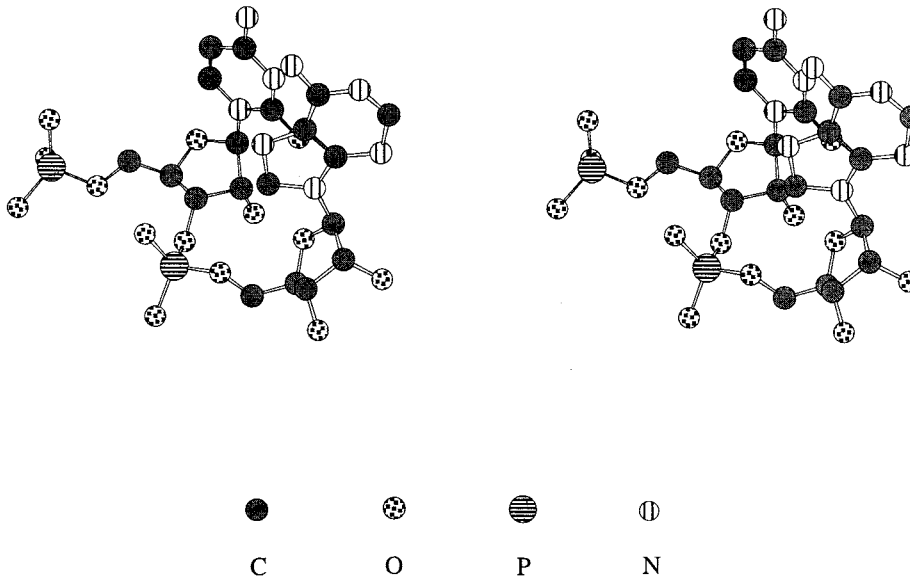

Fig. 5. A stereoview of part of a hammerhead ribozyme [23], in the neighborhood of the scissile bond. The incoming 2′-hydroxyl group, the phosphorus, and the 5′-oxygen leaving group are not in-line.

hammerhead ribozyme a magnesium ion was reported to interact directly with the unesterified *pro*-R oxygen of the starting phosphate diester [27].

The apparent contradiction of an adjacent setup but a reaction that went with inversion led several workers to suggest [22,23,28] that before the 2′-hydroxyl attacks the phosphorus, the hammerhead ribozyme must undergo a conformational change, away from the observed adjacent setup, so that the leaving group and the nucleophile are brought more nearly in-line with the phosphorus atom. Indeed, such a conformational change subsequently was reported [29] for a modified hammerhead ribozyme. In this modified ribozyme, the product-formation step was slowed by substitution of a methyl for a hydrogen on the 5′-methylene of the nucleoside that is displaced (A-1.1). It is not known to what extent this conformational change applies to the unmodified ribozyme, although a smaller conformational change had already been detected by freeze trapping the RNA prior to cleavage [27]. In a recent paper, it was reported [30] that the position of C17 in the ribozyme-cyclic phosphate product has undergone a considerably greater positional change than would be needed to just bring the departing 5′-oxygen in-line with the phosphorus and the 2′-hydroxyl of C17.

The calculations presented above suggest that if a magnesium ion were present, and in the correct position to stabilize an apical oxyanion, then inversion of configuration in phosphorus still could have resulted from adjacent attack. Thus a conformational change of the normal hammerhead ribozyme prior to reaction would not be an obligatory step. The conformational changes that have been seen in the crystal structures [27,29,30] are not necessarily inconsistent with adjacent attack for the normal ribozyme reaction. Clearly, the *final* arrangement of nucleophile, phosphorus and leaving group must be in-line just before breakdown, and one would therefore expect this geometry to be reachable without great difficulty. The question is the timing of the conformational change. A recent molecular dynamics simulation of the hammerhead did not reach an in-line conformation, although when the sugar pucker at C17 was forced to C2′-endo from its normal C3′-endo, the phosphorus was found to approach closer to the 2′-hydroxyl group [31].

However, the likelihood that a metal ion is directly involved in the catalytic action of this ribozyme has been seriously questioned in two recent papers [32,33]. Also it has been shown in these and in an earlier paper [34] that high concentrations of Na^+, Li^+, or NH_4^+ (or to some extent even the exchange-inert $Co(NH_3)_6^{3+}$) can replace the magnesium in the hammerhead ribozyme reaction. Univalent cations including sodium, potassium, or amines, can accelerate displacement reactions at a phosphate diester [35,36], and it is possible that molar concentrations of these same ions could stabilize a phosphorane oxyanion in an apical position, and allow adjacent attack. We acknowledge that in-line attack followed by in-line departure is a simpler mechanism, but the purpose of this paper is to emphasize that as long as something is able to stabilize an apical $P–O^-$, then adjacent attack followed by in-line departure should not be dismissed solely on the basis that the stereochemistry in phosphorus is inverted during the reaction. It is of course possible that the mechanism could change from adjacent attack to in-line attack upon changing the cation, for the conformational change presumably still is accessible.

It has been reported [11] that accelerated cleavage between C138 and A139 of P5abc RNA (from the *Tetrahymena thermophila* Group I intron) occurs in the absence of

magnesium, but that the addition of 20 mM magnesium suppresses cleavage at this bond. Soukup and Breaker postulate that cleavage in the absence of magnesium may be because the setup is correct for in-line attack [37], and they suggest that the local structure near this bond may not be very different in the presence and in the absence of magnesium because accelerated cleavage at adjacent G · A base pairs is seen under both sets of conditions. In the presence of magnesium setup is indeed close to in-line [37], although the distance from the phosphorus to the 2′-oxygen is about 3.5 Å, rather than the 3.0 Å suggested as ideal by Soukup and Breaker.

It has been shown recently [38] that a truncated version of the P5abc RNA has an extended conformation in the absence of magnesium but can take up a more compact conformation (“folded”) that has more tertiary structure when magnesium is present. Tinoco and coworkers show C138 base-paired to G180 in both the folded and extended forms, but they also show G164 shifting its pairing from A139 in the folded form, to U177 in the extended form. It is therefore possible that the addition of magnesium changes the conformation near C138 more than Soukup and Breaker had realized. Our calculations allow an alternative explanation for the lack of accelerated cleavage in the folded form, in that we have shown that the presence of magnesium *de*stabilizes the TBP for in-line attack. Relevant to this, Cate and Doudna [39] have reported that C138 is directly adjacent to a metal-ion binding site.

4. Future directions

We emphasize that although inversion could be consistent with adjacent attack, it certainly does not require it, and there are some unanswered questions that would have to be addressed if adjacent attack were to be preferred over in-line attack for the action of a ribozyme. Chief among these are why the intermediate [2′,O] should move towards [3′,S] but not towards [3′,OR] (which might have broken down to give the wrong cyclic phosphate), and why the trigonal bipyramid [3′,S] does not break down by departure of the apical 3′-O to give a 2′,5′-internucleotide bond. Also, in the case of a phosphorothioate, the effect of replacing magnesium by manganese would need to be reconsidered [40,41]. Clearly the details of proton transfers and the positioning of the magnesium ions will have a major influence on which bonds are made or broken. However, we hope that when an adjacent setup is encountered and inversion proved, all possibilities still will be considered.

Acknowledgments

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