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Ribozyme-catalyzed trimming reactions and the direct role of Mg^{2+} ions in the cleavage of RNA

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Summary

In the hypothetical RNA world divalent Mg^{2+} ions were exploited for cleavage (or ligation) of ribonucleic acids. Although some Mg^{2+} ions are involved in forming the tertiary structures of RNAs, the key Mg^{2+} ions are directly involved in catalysis not only in the case of hammerhead ribozymes but also in the case of *Tetrahymena* and, possibly, other types of ribozyme. RNA components bind the indispensable Mg^{2+} ions to the phosphodiester bonds that are being broken (or formed). Our analysis indicates that the chemical cleavage step of reactions catalyzed by the hammerhead ribozyme does not appear to have been perfected and, thus, it seems possible to create RNA-cleaving agents that are significantly more active than the standard hammerhead ribozyme. Moreover, RNA-cleaving mechanisms might converge as one unique and universal mechanism, exploited not only by various kinds of ribozyme but also by artificially created metal-ion-dependent DNAzymes and other RNA-cleaving agents that are yet to be identified.

I. Introduction

The term "ribozyme" is derived from the terms ribonucleic acid (RNA) and enzyme, and denotes a type of RNA molecule with catalytic properties. Researchers used to believe that RNA merely acted as an intermediary in the process of genetic-information transfer from DNA to protein molecules. It was not until the publication of work by Altman and Cech that RNA was shown also to play a catalytic role in the cell (Altman, 1989; Cech, 1990). A number of other natural ribozymes and *in vitro* selected ribozymes (Altman, 1989; Noller et al., 1992; Piccirilli et al., 1992; Symons, 1992; Bartel and Szostak, 1993; Lorsch and Szostak, 1994; Dai et al., 1995; Wilson and Szostak, 1995; Zhang and Cech, 1997; Vaish et al., 1998) have been discovered since the original discoveries of RNase P and the *Tetrahymena* ribozyme, but from certain standpoints one of the most important discovery has been that of the hammerhead ribozyme (Symons, 1992; Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Sarver et al., 1990; Eckstein and Lilley, 1996; Turner, 1997; Scanlon, 1997; Zhou and Taira, 1998). This ribozyme, first developed by an Australian research team, can act within a single molecule (*cis*-acting) but has also been engineered in such a way that it acts against other molecules (*trans*-acting) as well (Symons, 1992; Haseloff & Gerlach, 1988).

Until the discovery of ribozymes, attempts to fathom the origin of life were plagued by the classic chicken-and-egg paradox -- did nucleic acids carrying the genetic information required to make proteins come first, or did proteins that could synthesize nucleic acids come first? The problem with the hypothesis of protein primacy is that even if one assumes that amino acids could join randomly with one another to form functional proteins, proteins lack a suitable mechanism for transmitting the information on how amino acids should join up to make the next generation of proteins. The discovery of ribozymes, which have enzymatic activities and, at the same time, are capable of carrying genetic information, strongly suggests that nucleic acids, in the form of RNA, were responsible for the origin of life (Gesteland and Atkins, 1993). New research is demonstrating that in several functionally important enzymes, in addition to RNase P, which consist of protein and RNA, the RNA component is in fact, the source of the enzymatic activity (Noller et al., 1992; Sawa and Shimura, 1992; Nitta et al., 1998). Scientists can no longer deny the potential key role of RNA in the origin of life, and new research is constantly revealing that RNA plays a more significant role than anticipated in all life processes (Kobayashi et al., 1993, 1995).

At first, we initiated a program of basic research into ribozymes by undertaking molecular orbital calculations (Taira et al., 1990a, 1991a, 1993; Uchimaru et al., 1991, 1992; Storer et al., 1991; Yliniemela et al., 1993; Uebayasi et al., 1994; Zhou and Taira, 1998), and the calculations hint at the positive role of the magnesium ion in catalysis (Uebayasi et al., 1991; Uchimaru et al., 1993). That is, in both *Tetrahymena* and hammerhead ribozymes, magnesium ions may act as true catalysts. These findings indicated that ribozymes are essentially metallo-enzymes like many other protein enzymes and that the magnesium ions play pivotal roles in catalysis rather than just maintaining the tertiary structures of RNA components (Uebayasi et al., 1991; Uchimaru et al., 1992, 1993; Eckstein and Lilley, 1996; Zhou and Taira, 1998). In agreement with this finding many groups have been able to replace significant portions of hammerhead ribozymes by DNA components (Perreault et al., 1990, 1991; Yang et al., 1990, 1992; Pieken et al., 1991; Williams et al., 1992; Taylor et al., 1992; Paolella et al., 1992; Goodchild et al., 1992; McCall et al., 1992; Hendry et al., 1992; Nishikawa et al., 1991; Taira and Nishikawa, 1992; Shimayama et al., 1992, 1993; Warashina et al., 1997). The groups of Rossi and Jennings, as well as our own, have found that it is also possible to engineer a chimeric DNA/RNA ribozyme that is a better cleaver of RNA than the analogous all-RNA ribozyme (Taylor et al., 1992; Hendry et al., 1992; Shimayama et al., 1992, 1993; Warashina et al., 1997). Moreover, several attempts to generate DNA enzymes by *in vitro* selection have been successful and single-stranded DNA molecules with enzymatic activity have been isolated, including a Pb^{2+}-dependent DNAzyme with RNA-cleavage activity by Joyce's group (Breaker and Joyce, 1994), a DNAzyme with ligase activity by Szostak's group (Cuenoud and Szostak, 1995), and a DNAzyme with self-cleaving activity by Breaker's group (Carmi et al., 1996). Almost all of the DNAzymes isolated to date by *in vitro* selection require metal cofactors, such as Pb^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+} or Ca^{2+} ions (Breaker and Joyce, 1994; Breaker and Joyce, 1995; Faulhammer and Famulok, 1996; Santoro and Joyce, 1997). The Mg^{2+}-binding motif used in the RNA world appears to be conserved even in some of the DNAzymes as is conserved in the protein world (Beese and Steitz, 1991; Steitz and Steitz, 1993).

In this report we will review trimming reactions catalyzed by a hammerhead ribozyme, summarizing our data on transcribed ribozymes, and the results of our molecular orbital calculations; finally we suggest that the mechanisms might converge as one unique and universal mechanism, exploited not only by various

kinds of ribozyme but also by artificially created metal-ion-dependent DNAzymes and other RNA-cleaving agents that are yet to be identified.

II. Results and Discussion

A. Rates of transcription and cleavage are similar for *cis*-acting hammerhead ribozymes

We previously constructed a novel transcription system that allows trimming of both the 5' and the 3' termini of any RNA transcript by a *cis*-acting ribozyme (Taira et al., 1990b, 1991b; Yuyama et al., 1992; Ohkawa et al., 1993). The vector consists of a promoter, the "5' Processing Ribozyme", the DNA template (any DNA) to be transcribed, and the "3' Processing Ribozyme" (the pGENE8459 Series of vectors, **Fig. 1**). In our original construct, designated pGENE8459v3, a *trans*-acting ribozyme (called "Ribozyme for *SFL*1"; **Fig. 2, *Left***) was placed between the "5' Processing Ribozyme" and the "3' Processing Ribozyme" (Taira et al., 1991b). To characterize self-processing reactions during transcription and, in particular, to estimate the relative rates of both reactions, the RNAs obtained as products of transcription of the covalently closed circular (nonlinearized), as well as *Hind*III-linearized, pGENE8459v3 plasmids were analyzed at several time points (**Fig. 3**). When the RNA transcripts from the *Hind*III-linearized pGENE8459v3 were examined (after run-off transcription), six products were obtained. The various bands represent the following:

Band 1: Initial run-off transcription product (173 nt).
Band 2: Product of partial cleavage, produced by the action of the "5' Processing Ribozyme" (120 nt).
Band 3: Product of partial cleavage, produced by the action of the "3' Processing Ribozyme" (114 nt).
Band 4: Final product (Ribozyme for *SFL*1; 61 nt)
Band 5: Final product (3' Processing Ribozyme; 59 nt)
Band 6: Final product (5' Processing Ribozyme; 53 nt)

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Figure 1. The pGENE8459 series of vectors for transcription of RNA. Any DNA template can be inserted between two *cis*-acting ribozyme sequences. The first ribozyme, called "5' Processing Ribozyme", trims the 5'-region of the transcribed inserted gene and, similarly, the second ribozyme, called "3' Processing Ribozyme", trims the 3'-region of the transcribed RNA. It is, thus, not necessary to linearize the DNA template in order to obtain RNA transcripts with defined 5' as well as 3' ends.

Figure 2. Structure of the pGENE8459v3 vector. The "5'-Processing Ribozyme" has a second binding site (right) which is inactive in terms of 5'-end trimming.

Figure 3. Kinetics of self-cleavage and transcription reactions. RNA transcripts were obtained by transcription from the same amount of either circular or *Hind*III-linearized pGENE8459v3 template. Sampling was performed 30, 60, 140, and 360 min after initiation of the transcription reaction.

The initial full-length RNA transcript of 173 nucleotides (nt), consisting of "5' Processing Ribozyme", "Ribozyme for *SFL*1", and "3' Processing Ribozyme" (band 1), is ultimately cleaved spontaneously into three fragments (bands 4-6) by the action of both the "5' Processing Ribozyme" and the "3' Processing Ribozyme". Two fragments arising from partial digestion of the full-length RNA are also discernible: the first one (band 2) is a faint band of 120 nt consisting of "Ribozyme for *SFL*1" and the "3' Processing Ribozyme" produced by the action of the "5' Processing Ribozyme"; and the second one (band 3) is a distinct band of 114 nt consisting of the "5' Processing Ribozyme" and "Ribozyme for *SFL*1" which was produced from the full-length transcript by the action of the "3' Processing Ribozyme".

It is important to note that, despite the equimolar amount of linearized pGENE8459v3 template, more than 10 times higher level of RNA product (band 4) was produced from the DNA template (insert) in **Figure 1** when the circular template was used: note the more prominent 61 nt band generated from the circular template. Therefore, the efficiency of transcription is much higher for the circular template, possibly because (i) RNA polymerase prefers a circular template over a linearized template (when both circular and linearized pGENE8459v3 DNAs were mixed and used as templates, almost no products of transcription arising of the linearized template were observed); and (ii) with the circular template, "rolling-circle" transcription is possible, which circumvents kinetically inefficient diffusion-controlled association/ dissociation processes.

The type of vector used here is particularly suitable for preparation of uniform RNAs; *e.g.*, for NMR measurements or for crystallization. It is known that DNAs with heterologous 5' or 3' ends hinder crystallization. Similarly, RNAs with heterologous ends are expected to hinder crystallization. When our constructs are used, uniform RNAs with defined 5' and 3' ends can be produced. Indeed, our trimming vector has been proven to be extremely useful for the synthesis of short RNAs (Price et al., 1995). Moreover, it is possible to concatenate entire units in tandem. When 10 units are concatenated, the yield of RNA transcripts increases 10-fold, as compared to the results of transcription from a DNA template with only one unit (of course, in both transcriptions, equimolar amounts of DNA template were used). In addition, different types of RNA sequence can be produced, depending on the kind of insert in the "DNA Template" region indicated in **Figure 1** (Ohkawa et al., 1993; Price et al., 1995). Therefore, by concatenating several units, each of which contains a different "DNA Template", we can produce several types of RNA. This methodology is especially useful when ribozymes are to be used against HIV, because HIV is infamous for its high frequency of mutation, which incidentally poses problems for the immune system that is already depressed after HIV-1 infection. This genetic polymorphism not only makes it far more difficult than might be anticipated to find a vaccine for HIV-1 but also poses a challenge to the use of ribozymes as a form of treatment. Although a ribozyme has high sequence-specificity, once the nucleotide sequence in the target RNA chain has been altered, the ribozyme can lose its effectiveness. Nonetheless, although HIV does exhibit such genetic variability, this variability is not limitless. Changes do not occur as frequently in those sections of the RNA chain that code for significant viral functions. Ribozymes that are targeted simultaneously to a number of these highly conserved and less mutable sites should prove to be effective anti-viral agents. Even if one or two of the sites were altered, as long as one or more unaltered sites remained, these sites would be attacked by ribozymes, with resultant inactivation of the functional virus. The probability that every one of the functionally significant sites would undergo simultaneous alteration is extremely low, if not zero. A detailed description of this system of treatment of HIV infection is, however, beyond the scope of this paper.

Having characterized the properties of our vector, we can now return to **Figure 3** and analyze the rates of transcription and cleavage reactions. Examination of the intensities of the bands from the linearized template (bands 1-6) allows us to conclude that the rates of the transcription and cleavage reactions *in vitro* are similar. The initial product of transcription (band 1) undergoes self-cleavage to produce the partially digested products (bands 2 and 3). If the rate of transcription were much higher than the rate of cleavage, one would expect an increase in the intensity of band 1. However, if the rate of cleavage were much higher than the rate of transcription, one would expect more intense bands of the completely cleaved products (bands 4-6) with almost no intermediates (bands 2 and 3). Since all six bands are recognizable, the rate of transcription and the rate of cleavage must be similar. Nonetheless, a comparison of bands 2 and 3 reveals that the "3' Processing Ribozyme" appears more active than the "5' Processing Ribozyme", because the intensity of band 2 is much lower than that of band 3. Note here that the material in band 2 was degraded into "Ribozyme for *SFL*1" (band 4) and "3' Processing Ribozyme" (band 5) by the action of the "3' Processing Ribozyme". However, we now find that the "5' Processing Ribozyme" has a second binding site, and that it forms an inactive complex with respect to the cleavage reaction (see the right hand panel of **Fig. 2**). It is important to note that, in general, ribozymes tend to form inactive complexes when there exists an alternative binding site. Therefore, care must be taken in choosing the target sequence of a

ribozyme. In fact, the removal of the second binding site (removal of the *Sac*I site) accelerated the cleavage of the intermediate (band 3; data not shown). Therefore, the actual rate of cleavage is higher than the anticipated rate based on the intensity of band 3 in **Figure 3**.
The results described above mean that the cleavage of the *cis*-acting hammerhead ribozyme occurs more rapidly or at least at a rate similar to the rate of transcription *in vitro*. Since natural hammerhead ribozymes act in *cis* during replication by the rolling-circle mechanism (Symons, 1992), there has been no selective advantage to further improvements in the chemical-cleavage step, with respect to its natural function. So it is possible that the active site of the hammerhead ribozyme has not been perfected. In fact, we obtained a chimeric RNA/DNA ribozyme which has much higher activity (under extreme conditions with the cleavage rate constant of 100 min^{-1}; Shimayama et al., 1995) than a natural hammerhead ribozyme (that usually has the cleavage rate constant of about 1 min^{-1}), supporting the possibility to engineer the natural ribozyme or select *in vitro* of artificial RNA-cleaving agents that are better cleavers of RNAs.

B. Ribozymes are metallo-enzymes: the Mg^{2+}-binding motif of RNA-cleaving agents

Here we describe the binding motif of Mg^{2+} ions. The molecular orbital calculations that we will discuss in this section strongly support a more direct role for the Mg^{2+} ion as the real catalyst in RNA-cleavage reactions. Since we are interested in the energetics in RNA-cleavage reactions, we have carried out *ab initio* molecular orbital calculations using several model compounds (Taira et al., 1990a, 1991a, 1993; Uchimaru et al., 1991, 1992; Storer et al., 1991; Yliniemela et al., 1993; Uebayasi et al., 1994; Zhou and Taira, 1998), and the calculations hint at a positive role for the Mg^{2+} ion in catalysis (Uebayasi et al., 1991; Uchimaru et al., 1993; Uebayasi et al., 1994; Zhou and Taira, 1998). Moreover, we analyzed *Tetrahymena* ribozyme reactions and the quantitative details have been published elsewhere (Uchimaru et al., 1993). In this section we will discuss the role of Mg^{2+} ion qualitatively.
In *Tetrahymena* ribozyme reactions, the transesterification reaction is initiated by the attack of the 3'-hydroxyl group of the bound guanosine (G) on a phosphodiester linkage to generate the cleaved upstream exon with a 3'-hydroxyl group and an intron with 5'-G (**Fig. 4**). Divalent magnesium ions are commonly indispensable as cofactors for the self-cleavage of phosphodiester linkages in ribozyme-catalyzed reactions. The self-splicing reactions proceed with inversion of the configuration at the phosphorus center and, thus, an *in-line* mechanism ($S_N2(P)$ process) appears the most likely (McSwiggen et al., 1989; Rajagopal et al., 1989). Consequently, a pentacoordinate oxyphosphorane intermediate/ transition state is postulated for consecutive transesterification reactions of the *Tetrahymena* rRNA splicing process (structure shown in parentheses in **Fig. 4**).
We have analyzed the electrostatic potential, which represents the energy of interaction between a positive charge and a negative charge of a molecule. Thus, the electrostatic potential should be useful for interpretation of ionic interactions (Uchimaru et al., 1993; Uebayasi et al., 1994). In our model system, we examined the interaction between dianionic trimethoxyphosphorane and the Mg^{2+} ion (overall, a neutral complex). As shown in parentheses in **Figure 4**, in the transition state for the transesterification process, most negative charges (up to two charges) are localized on the non-bridging phosphoryl oxygens and this observation appears to support the coordination of a Mg^{2+} ion between these two non-bridging oxygens. However, our molecular orbital calculations indicate, instead, that the Mg^{2+} coordination occurs preferably in the region between the bridging and non-bridging oxygens, as indicated by the shaded areas in **Figure 5A** and as also depicted by the circled Mg^{2+} ions in **Figure 4** (Uchimaru et al., 1993). This conclusion is consistent with the recent findings that the *Tetrahymena* ribozyme has two metal ions at its catalytic center and that each metal ion interacts with the leaving 3'-oxygen (Piccirilli et al., 1992) and the attacking 3'-hydroxyl group of the bound guanosine (G) (Weinstein et al., 1997), respectively.
The more symmetrical transition state of the catalytic center of the *Tetrahymena* ribozymes, as compared to that of the hammerhead ribozyme, makes it easier to carry out expensive molecular orbital calculations. In nature, the symmetrical transition state of the *Tetrahymena* ribozyme can be used not only to cleave the bonds but also to ligate the bonds. In fact, the *Tetrahymena* ribozyme has been shown to have polymerase activities (Been and Cech, 1988; Weinstein et al., 1997). Since the *Tetrahymena* ribozyme does not utilize the 2'-oxygen on the ribose ring of the cleavage site, it can even cleave DNAs (Hershlag and Cech, 1990; Robertson and Joyce, 1990). This kind of property of *Tetrahymena*-type ribozymes could be advantageous during the development of the DNA world.

A similar electrostatic potential analysis of a hammerhead ribozyme indicates that the Mg^{2+} coordination occurs preferably in the region between the bridging and non-bridging oxygens, as indicated by the shaded areas in **Figure 5B**. Further analysis demonstrates that magnesium ion itself is capable of cleaving (or forming from the principle of microscopic reversibility) of a phosphorus-oxygen bond by a direct coordination to the translating oxygen (Uebayasi et al., 1994). In this scenario, the direct coordination of the metal ion with the 2'-oxygen of the attacking nucleotide residue, as shown in **Figure 6**, polarizes and weakens the 2'-OH bond. As a result, higher concentrations of the active nucleophile, the metal-bound-2'-alkoxide of the ribose, becomes available. Therefore, an inverse correlation between the pK_a of the metal-bound ribose 2'-OH and the ribozyme activity holds. In other words, the lower is the pK_a of the metal ion, the higher is the cleavage rate at a given concentration of the metal ion at a fixed pH. Similarly, the direct coordination of the metal ion, that acts as a Lewis acid, with the 5'-oxygen of the leaving nucleotide residue weakens the P-(5'-O) bond. Metal ions with lower pK_a values will weaken the phosphorus-(5'-oxygen) bond to a greater extent, thereby, activating the ribozyme-mediated cleavage to a greater extent. This kind of metal-ion-binding motif utilized by the *Tetrahymena* and hammerhead ribozymes, in which the Mg^{2+} ions coordinate directly with the attacking and leaving oxygens, appears to be conserved even in the protein world. DNA polymerase I from *E. coli* is a metallo-enzyme and it uses two Mg^{2+} ions (Beese and Steitz, 1991; Steitz and Steitz, 1993): the coordination sites of these Mg^{2+} ions are between the bridging and non-bridging oxygens, in agreement with the results of calculations shown in **Figure 5**.

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Figure 4. Splicing reactions of the *Tetrahymena* pre-rRNA. Chemical structures for the splicing reactions are shown at the bottom. The structure of possible transition state structure is depicted in parentheses.

Figure 5. The side view of a three-dimensional representation of the surface of constant electrostatic potential for trimethyloxyphosphorane (**A**), a model for the transition state structure of the *Tetrahymena* ribozyme shown in parentheses in **Figure 4**, and for methylethylene oxyphosphorane (**B**), a model for the transition state structure of a hammerhead ribozyme shown in **Figure 6**. The shaded areas represent the regions with most negative electrostatic potential. These regions are the most favorable sites for the coordination of Mg^{2+} ions. Note that, although dianionic oxyphosphorane concentrates its negative charges on the non-bridging phosphoryl oxygens, the coordination of an Mg^{2+} ion between these two non-bridging oxygens is unlikely.

III. Conclusion

We suggest that the catalytic center of the hammerhead ribozyme has not been perfected for chemical-cleavage reactions because, in its natural role as a *cis*-acting ribozyme, there has not been strong evolutional pressure towards such perfection since the chemical step appears to be more rapid than the replication processes. Therefore, it seems possible to improve the chemical-cleavage step by, for example, the use of *in vitro* and *in vivo* selection procedures or combinatorial chemistry. Molecular orbital calculations predict that a Mg^{2+} ion does not bifurcate between the most negatively charged non-bridging oxygens in the transition state of the transesterification reactions and, instead, the preferred Mg^{2+}-coordination site is in the space between the bridging and non-bridging oxygens, where Mg^{2+} ions can act as Lewis acid catalysts, facilitating the formation and cleavage of phosphorus-oxygen bonds. Finally, RNA-cleaving mechanisms might converge as one unique and universal mechanism, exploited not only by various kinds of ribozyme but also by artificially created metal-ion-dependent DNAzymes and other RNA-cleaving agents that are yet to be identified.

IV. Experimental procedures

A. Multitarget-ribozyme expression plasmid (the pGENE8459 series of vectors) and cleavage activities of transcribed ribozymes

As described previously, sequences of the various constructed plasmids were confirmed using a DNA Sequencer (model 373A; Perkin Elmer, Applied Biosystems, Foster City, CA, Taira et al., 1991b, 1992). Transcription was carried out in a total volume of 25 μL of solution that contained 2 μL of 5x transcription buffer (1x = 200 mM Tris-HCl, pH 7.5; 30 mM $MgCl_2$; 10 mM spermidine; 0.05% bovine serum albumin); 1.25 μL of 0.2 M DTT; 2.5 μL of NTP mix (500 μM each of UTP, ATP, CTP, and GTP); 1.25 μL of human placental ribonuclease inhibitor (20 units/μL; Toyobo, Tokyo); 0.5 μL of [α-^{32}P] CTP (20 μCi/μL, ~800 Ci/mmol); 2.5 μL of template DNA solution (pGENE8459v3 or pGENE8459v3 with the *Sac*I site removed; 1 μg/μL); and 0.65 μL of T7 RNA polymerase (20 units/μL; Amersham, Tokyo). Transcription reactions were carried out and kinetics were analyzed at 37°C. The products of transcription and cleavage were analyzed by reference to sequencing ladders of pGENE8459v3 on a 6% polyacrylamide gel that contained 8.3 M urea.

Figure 6. The double-metal-ion mechanism of catalysis for reactions catalyzed by hammerhead ribozymes (Steitz and Steitz, 1993; Uebayasi et al., 1994; Sawata et al., 1995; Pontius et al., 1997; Zhou et al., 1997; Lott et al., 1998; Zhou and Taira, 1998).

B. Molecular orbital calculations

GAUSSIAN 88 (Frish et al, 1988) and GAUSSIAN 90 (Frish et al, 1990) program packages were used for geometry optimizations and analyses of Mulliken populations and natural bond orbitals (NBO). SPARTAN 90 (Carpenter et al, 1990) generated the three-dimensional representations of molecular structures and electrostatic potentials. All the calculations in the present work were performed at the Hartree-Fock level. Dianionic trimethoxyphosphorane (**Fig. 5A**) , which is a model for the reaction center of the *Tetrahymena* ribozyme, and the locations of Mg^{2+} ions relative to the trimethoxyphosphorane were geometrically optimized. In addition, electrostatic potential calculations for the trimethoxyphosphorane dianion were performed using self-consistent-field (SCF) densities. For this purpose the 6-31G* optimized structure was used. Further details of the procedures are available elsewhere (Uchimaru et al., 1993). Similarly, dianionic cyclic phosphorane (**Fig. 5B**) was used as a model compound for the transition state of a hammerhead ribozyme (Uebayasi et al., 1994).

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