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Metal Ion Requirements for Sequence-Specific Endoribonuclease Activity of the *Tetrahymena* Ribozyme†

Cheryl A. Grosshans and Thomas R. Cech*,‡
Howard Hughes Medical Institute, Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215
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ABSTRACT: A shortened form of the self-splicing intervening sequence RNA of *Tetrahymena thermophila* acts as an enzyme, catalyzing sequence-specific cleavage of RNA substrates. We have now examined the metal ion requirements of this reaction. Mg^{2+} and Mn^{2+} are the only metal ions that by themselves give RNA enzyme activity. Atomic absorption spectroscopy indicates that Zn, Cu, Co, and Fe are not present in amounts equimolar to the RNA enzyme and when added to reaction mixtures do not facilitate cleavage. Thus, these ions can be eliminated as cofactors for the reaction. While Ca^{2+} has no activity by itself, it alleviates a portion of the Mg^{2+} requirement; 1 mM Ca^{2+} reduces the Mg^{2+} optimum from 2 to 1 mM. These results, combined with studies of the reactivity of mixtures of metal ions, lead us to postulate that two classes of metal ion binding sites are required for catalysis. Class 1 sites have more activity with Mn^{2+} than with Mg^{2+}, with the other divalent ions and Na^+ and K^+ having no activity. It is not known if ions located at class 1 sites have specific structural roles or are directly involved in active-site chemistry. Class 2 sites, which are presumably structural, have an order of preference $Mg^{2+} \geq Ca^{2+} > Mn^{2+}$ and $Ca^{2+} > Sr^{2+} > Ba^{2+}$, with Zn^{2+}, Cu^{2+}, Co^{2+}, Na^+, and K^+ giving no detectable activity over the concentration range tested.

Some protein enzymes bind specific metal ions and use them for catalysis. Examples of metalloenzymes are carboxypeptidase A, carbonic anhydrase, alkaline phosphatase (Zn^{2+}), staphylococcal nuclease (Ca^{2+}), cytochrome oxidase (Cu^{2+}/Cu^+ and Fe^{4+}/Fe^{3+}), and xylose isomerase (Mg^{2+} or Mn^{2+}) (Coleman & Gettins, 1983; Fersht, 1985; Stryer, 1988; Farber et al., 1989). Metal ions also contribute to RNA catalysis. Group I and group II introns have an absolute requirement for either Mg^{2+} or Mn^{2+}, as does ribonuclease P [reviewed by Cech and Bass (1986)]. Another group of RNA enzymes derived from plant viral satellite RNAs are greatly stimulated by a variety of divalent and polyvalent cations (Prody et al., 1986; Uhlenbeck, 1987).

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* Author to whom correspondence should be addressed.
‡ American Cancer Society Professor and Investigator, Howard Hughes Medical Institute.

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Interpretation of metal ion requirements is much more complicated for RNA catalysis than for protein catalysis because RNA molecules, unlike most proteins, require divalent cations for formation of their native structures. The metal ions act as counterions for the polyanionic nucleic acid and, in addition, bind to specific sites to stabilize specific nucleic acid structures (Fresco et al., 1966; Holbrook et al., 1977; Jack et al., 1977). Because metal ions determine structure and structure determines function, the requirement of a divalent metal ion for RNA catalysis does not by itself imply its direct involvement in active-site chemistry. Nevertheless, proposals that metal ions in catalytic RNAs serve to deprotonate a nucleophile, activate an electrophile, or stabilize an anionic transition state are attractive and have been the subject of considerable discussion (Brown et al., 1983; Haydock & Allen, 1985; Gardiner et al., 1985; Guerrier-Takada et al., 1986; Cech, 1987).

We have now studied the metal ion requirements for a nucleotidyl transfer reaction catalyzed by the *Tetrahymena* ribozyme, an RNA enzyme derived from a group I intron. In this reaction, the *Tetrahymena* ribozyme acts as a sequence-specific endoribonuclease or "RNA restriction enzyme", cleaving an oligoribonucleotide substrate with concomitant addition of guanosine to the 5′ end of the downstream cleavage product (Zaug et al., 1986).

Materials and Methods

Materials. Labeled nucleoside triphosphates were purchased from either New England Nuclear (NEN) or ICN, unlabeled nucleosides and nucleoside triphosphates from P-L Biochemicals, calf intestinal phosphatase from NEN, T_4 polynucleotide kinase from United States Biochemicals, and DNA restriction endonucleases from New England Biolabs. T_7 RNA polymerase was isolated from *Escherichia coli* strain BL21, containing plasmid pAR1219 (Davanloo et al., 1984). Ultrapure $MgCl_2$ was purchased from Johnson Matthey Aesar Group, ultrapure Tris base from Bio-Rad, and ultrapure Tris-HCl from Schwarz/Mann Biotech. Water was purified to 18 MΩ/cm with a Technic water purification system using reverse osmosis and deionization resins. Atomic absorption standard stocks were provided by Bob Meglen (Center for Environmental Services, University of Colorado, Denver). All stocks were made to 1000 ppm, except for Ca at 10 000 ppm, with 99.999% pure metals and 1% Ultrex nitric acid (J. T. Baker). Reagent metals were ACS reagent grade from J. T. Baker, EM Science, or Mallinckrodt.

Preparation of L - 21 ScaI Ribozyme. Plasmid pT7L-21 (Zaug et al., 1988) was digested with restriction endonuclease *Sca*I and then extracted with phenol and chloroform, ethanol-precipitated, and resuspended in water. L - 21 *Sca*I RNA was transcribed from 100 μg of pT7L-21 in 20 mL of 1 mM each nucleoside triphosphate, 15 mM $MgCl_2$, 40 mM Tris, pH 7.5, 2 mM spermidine, 5 mM DTT, and ~500 units of T_7 RNA polymerase/μg of plasmid DNA. The reaction was incubated at 37 °C for 2 h. RNA was ethanol-precipitated, resuspended, and purified by electrophoresis on two 3-mm-thick 4% polyacrylamide/8 M urea gels. Purified RNA was electroeluted from gel slices (Schleicher & Schuell Elutrap), extracted with phenol and chloroform, and ethanol-precipitated. It was then resuspended in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, and the concentration was determined spectrophotometrically. The reaction yielded a total of 4.4 mg of purified RNA, i.e., 44 μg of L - 21 *Sca*I ribozyme/μg of pT7L-21 plasmid DNA.

Preparation of Oligoribonucleotide Substrate. A 12-residue RNA substrate, GGCCUCUAAAAA, was transcribed from a synthetic DNA template by using a method similar to that described by Lowary et al. (1986) and Milligan et al. (1987). The product of a 1-mL transcription reaction was gel-purified and then resuspended in 100 μL of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Its concentration was determined by spectrophotometry. Substrate RNA was 5′ end-labeled by treatment with calf intestinal phosphatase followed by polynucleotide kinase and [γ-^{32}P]ATP. Labeled substrate was purified on a 0.5-mm-thick 20% polyacrylamide/8 M urea gel, visualized by autoradiography and retrieved from the gel slice. The substrate sequence was confirmed by enzymatic sequencing (Donis-Keller, 1977, 1980).

Endoribonuclease Assay. Reactions (20 μL) contained 50 mM Tris, pH 7.5, 0.5 mM GTP or guanosine, 0.5–10 mM $MgCl_2$, 2–15 μM substrate RNA, a trace quantity of 5′ end-labeled substrate RNA, and 0.2–3 μM L - 21 *Sca*I ribozyme and typically proceeded for 20 min at 50 °C. A 2× metal cocktail was used to start the reaction so that all metals were added to the reaction simultaneously. Reactions were stopped by addition of EDTA and urea to final concentrations of 50 mM and 7 M, respectively. They were analyzed by electrophoresis on 20% polyacrylamide denaturing gels followed by autoradiography. Quantitation was achieved by cutting the bands from the gel and counting in a liquid scintillation counter or by scanning the gel on the AMBIS radioanalytic system.

Atomic Absorption Spectroscopy. A Perkin-Elmer 360 spectrophotometer and HGA-2100 controller graphite furnace were used to analyze all of the components of the endoribonuclease reaction for specific trace metals. A typical determination involved injecting a 20-μL aqueous sample into a graphite tube and running the program which, for Zn, dried the sample at 125 °C for 20 s, charred for 10 s at 500 °C, and then atomized the sample at 2500 °C for 12 s. Similar optimized programs were used for Fe, Cu, Co, and Ca. As the samples were atomized, sharp peaks were recorded and their heights measured to quantify the metal of interest. Standard concentrations were calculated to read 0.2 absorbance unit, which for zinc is about 80 pg or 4 ppb. This corresponds to 5 pmol of zinc so that 20 μL of a 0.25 μM ribozyme mix would have the same absorbance as the standard sample if zinc were present in a 1:1 molar ratio with the ribozyme.

Method of Standard Additions. If no signal was observed for a specific component, a spike recovery was done in which 5 ppb standard was added to the component sample and to a blank and the two were analyzed side by side. A reduced peak height for the sample, which would indicate matrix suppression of the signal, was not observed for any of the components tested. If a reagent mixture produced a signal, as in the case of Zn and Fe, then the "Method of Standard Additions" (Skoog & West, 1980) was used to quantify metal levels.

Metal Contamination Precautions. Whenever possible, plasticware was used instead of glass. All plasticware and glass, including pipet tips, reaction tubes, reagent containers, and volumetric flasks, were soaked in 10% reagent-grade nitric acid for at least 48 h and then thoroughly rinsed with water from the Technic purification system. Water stored in and pipetted with acid-treated labware gave a metal signal which was only 5–20% that of water handled with autoclaved plasticware.

Results

Metal Ions as Stoichiometric Cofactors. It has long been known that either Mg^{2+} or Mn^{2+} is required for self-splicing

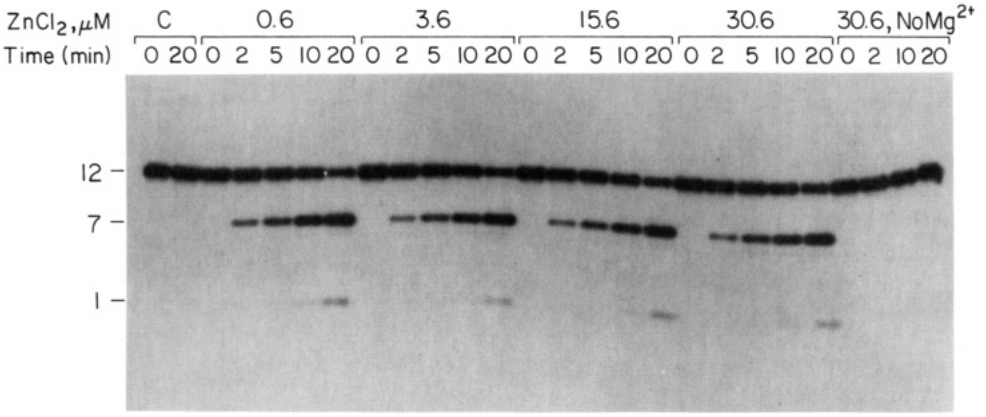

FIGURE 1: Rate of cleavage of the 12-nucleotide RNA substrate pGGCCUCUAAAAA by L - 21 *Sca*I ribozyme in 10 mM $MgCl_2$ and 0–30 μM added $ZnCl_2$. The reaction solution prepared with ultrapure reagents still contained 0.6 μM Zn^{2+}, as determined by atomic absorption spectroscopy. Each reaction contained 3 μM ribozyme, 15 μM RNA substrate, and 0.5 mM GTP. C, no ribozyme. The reaction temperature was 50 °C. The major product (7) is pGGCCUCU. The minor product (1) results from attack of the major product after the 5′-terminal pG of the substrate in a reaction analogous to exon ligation (Kay & Inoue, 1987; B. Flanegan and T. R. Cech, unpublished data).

and enzymatic activity of the *Tetrahymena* group I intron [reviewed by Cech and Bass (1986)]. Considering the ubiquity of trace amounts of other metal ions, however, a stoichiometric requirement for some additional metal ion might have been easily missed. For example, one could imagine that activity required stoichiometric Zn^{2+}. During purification, the ribozyme is subjected to denaturing gel electrophoresis through an EDTA-containing buffer solution, stripping it of metal ions. It is then ethanol-precipitated and redissolved, at which point it could pick up Zn^{2+} from the trace quantities present in reagent-grade chemicals, water, or plasticware. If the ribozyme is present in nanomolar concentration, it would not be difficult for it to obtain nanomolar Zn^{2+}. The ability to synthesize milligram quantities of the ribozyme (Zaug et al., 1988) allows direct analysis for metal ions and therefore a direct test of such a hypothesis.

An ultrapure ribozyme reaction solution was prepared by using reagents and techniques designed to minimize contamination by metal ions other than Mg^{2+} (see Materials and Methods). The solution contained 50 mM Tris buffer, pH 7.5, 10 mM $MgCl_2$, 0.5 mM GTP, 15 μM oligoribonucleotide substrate, and 3 μM L - 21 *Sca*I ribozyme. The high substrate and ribozyme concentrations were chosen to facilitate detection of any metal ions that might accompany these reagents.

The solution was analyzed for Zn, Cu, Co, Fe, and Ca by using atomic absorption spectroscopy. These metals were chosen because of their common occurrence in enzymes. Cu and Co were undetectable in the ribozyme reaction solution. The limit of detection of Co was determined to be between 0.3 and 0.8 μM. The limit of detection for Cu is reported to be 2 pg (Perkin-Elmer), and, in fact, a 1 μM Cu standard (1.3 ng) was easily detected although the limit was not determined. Zn was present at 0.56 ± 0.05 μM and Fe at 0.15 ± 0.08 μM, as determined by the standard addition method (see Materials and Methods). These concentrations are less than that of the ribozyme by factors of 5 and 20, respectively. [Essentially all of the ribozyme molecules are active, as indicated by active-site titration (J. McSwiggen and T. Cech, unpublished data).]

If any of these metal ions were required by the ribozyme in stoichiometric amounts, activity would be suppressed in the ultrapure reaction solution and might be markedly enhanced by the addition of the required metal. Therefore, $ZnCl_2$ and $Fe(NH_4)_2(SO_4)_2$ were added to the ultrapure solution at concentrations ranging from zero to a 10-fold molar excess over the ribozyme. Cleavage of the oligoribonucleotide substrate was then monitored. As shown in Figure 1, addition

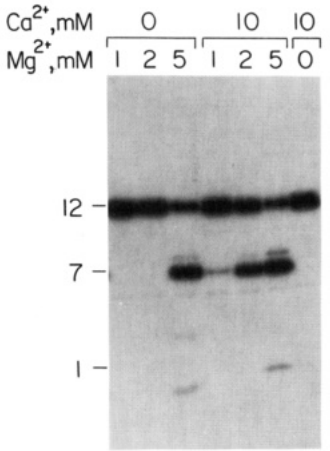

FIGURE 2: Endoribonuclease reaction at low $MgCl_2$ concentration in the presence and absence of $CaCl_2$. Substrate concentrations were 0.5 mM GTP and 15 μM RNA oligonucleotide with 3 μM ribozyme. Reactions proceeded for 20 min at 50 °C.

of Zn^{2+} did not affect the rate or specificity of the reaction. Equivalent results were obtained for Fe^{2+} (data not shown).

Calcium Ion. The fifth metal tested, Ca, was so ubiquitous that atomic absorption spectroscopy was abandoned. Instead, the Ca^{2+} chelator EGTA was employed to effectively remove Ca^{2+} from the ribozyme reaction solution. At an effective Mg^{2+} concentration of 10 mM, the reaction rate was indistinguishable in the presence and absence of EGTA. Furthermore, the addition of 1 mM $CaCl_2$ to 10 mM $MgCl_2$ did not change the rate (data not shown). However, when the Mg^{2+} concentration was reduced to 1 or 2 mM, activity was seen only upon the addition of $CaCl_2$. At 10 mM Ca^{2+} in the absence of Mg^{2+}, no activity was observed (Figure 2).

These measurements were done with GTP as the co-substrate. Because GTP is itself a metal ion chelator, the substitution of guanosine for GTP allowed activity to be observed with lower concentrations of both $MgCl_2$ and $CaCl_2$. All further references to metal ion dependence are based on experiments done with guanosine.

Kinetics as a Function of Mg^{2+} Concentration. The time course of the endoribonuclease reaction was determined in 1.0 mM $CaCl_2$ at varying Mg^{2+} concentration. Velocity, V, is plotted against $[Mg^{2+}]$ in Figure 3. The reaction does not follow a simple Michaelis–Menten model with respect to Mg^{2+} ion. The sigmoidal curve could indicate that Mg^{2+} binds cooperatively to the ribozyme. The concentration of RNA

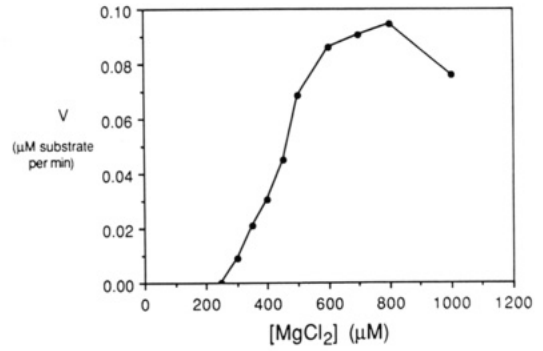

FIGURE 3: Velocity of the endoribonuclease reaction as a function of $MgCl_2$ concentration. All reactions contained 1 mM $CaCl_2$, 50 mM Tris, pH 7.5, 0.5 mM guanosine, 0.2 μM L - 21 *Sca*I ribozyme, and 5 μM substrate RNA. The temperature was 50 °C. Initial velocity was calculated as $V_0 = k_{obs}[S]$, where substrate RNA concentration, [S], was 5 μM and k_{obs} was determined from a linear regression of F, the extent of reaction, vs time. F vs time points were 2, 4, 6, 8, 12, 16, and 20 min.

phosphate in the reaction is calculated to be 138 μM, so Mg^{2+} is in 2-fold molar excess over RNA phosphate at the threshold Mg^{2+} concentration of 300 μM. Because each reaction contained 1 mM Ca^{2+} in addition to the Mg^{2+}, the total divalent cation is present in a larger molar excess.

Comparison of Divalent and Monovalent Cations. A side-by-side comparison of Zn^{2+}, Cu^{2+}, Co^{2+}, and Ca^{2+} at 0–10 mM confirmed that none of these metal ions conferred activity in the absence of Mg^{2+} and clearly indicated that only Ca^{2+} invoked any activity at 0.5 mM $MgCl_2$ (Figure 4). Similarly, the monovalent metal ions Na^+ and K^+ in concentrations ranging from 1 to 200 mM gave no activity in the presence of 0.5 mM Mg^{2+}. At 1 mM $MgCl_2$, the reaction rate with NaCl was significantly lower than that with $CaCl_2$ at an equivalent total concentration of cationic charge (Figure 5).

The reaction was inhibited at higher concentrations of Ca^{2+} or Na^+. A reaction containing 1 mM $MgCl_2$ was largely inhibited at 10–20 mM $CaCl_2$ (20–40 mM cationic charge) or at 20 mM NaCl, as shown in Figure 5. Analogous results were observed for this comparison at equivalent ionic strength (not shown).

No extensive degradation of the ribozyme occurred during reactions in the presence of these metal ions (data not shown), with the exception of Zn^{2+} at $[Zn^{2+}] \geq 1$ mM. The half-life of intact ribozyme was 10 ± 1 min in 1 mM $ZnCl_2$ and 5.2 ± 0.5 min in 10 mM $ZnCl_2$, measured under reaction conditions at 50 °C (not shown). Even in this case, the degradation of the ribozyme was not so rapid that it would have prevented detection of the endoribonuclease reaction.

Table I: Divalent Metal Combination Effects on Endoribonuclease Activity

row	$[Mg^{2+}]$ (mM)	$[Ca^{2+}]$ (mM)	$[Mn^{2+}]$ (mM)	[total cation] (mM)	fraction reacted in 20 min
1	1.5	0	0	1.5	0.79
2	1.0	0.5	0	1.5	0.66
3	0.5	1.0	0	1.5	0.40
4	0	1.5	0	1.5	0.00
5	0	0	1.5	1.5	0.57
6	0	0.5	1.0	1.5	0.69
7	0	1.0	0.5	1.5	0.79
8	1.5	1.0	0	2.5	0.69
9	1.5	0	1.0	2.5	0.69
10	0	1.0	1.5	2.5	0.80
11	1.0	0	1.5	2.5	0.62

Other Alkaline Earth Metals. Mg and Ca are group IIA alkaline earth metals. Two other group IIA metals, Sr and Ba, were tested as substitutes for Ca in the endoribonuclease reaction. Both metals stimulated activity in the presence of 0.5 mM $MgCl_2$. The order of rate enhancement was $Ca^{2+} > Sr^{2+} > Ba^{2+}$ (Figure 6).

Manganous Ion. Mn^{2+} behaves similarly to Mg^{2+} in reactions of the ribozyme. When used alone in the endoribonuclease reaction, 2 mM $MnCl_2$ generated only slightly less activity than the same concentration of $MgCl_2$. In conjunction with 1 mM Ca^{2+}, however, Mn^{2+} was more efficient than Mg^{2+}, showing optimal activity at 0.5 mM versus 1 mM for Mg^{2+} (Figure 7). Table I compares the activity of different combinations of Mg^{2+}, Mn^{2+}, and Ca^{2+}.

Discussion

Two Classes of Metal Ion Binding Sites. We have distinguished two classes of metal ion binding sites required for the endoribonuclease activity of the *Tetrahymena* ribozyme. Only Mg^{2+} and Mn^{2+} can promote the reaction by themselves, indicating a class of "activity" sites that must be filled by Mg^{2+} or Mn^{2+} to give an active ribozyme. The finding that Ca^{2+} can alleviate a portion of the Mg^{2+}/Mn^{2+} requirement indicates a second class of sites, which can be occupied by Ca^{2+}, Mg^{2+}, or Mn^{2+}. Because these sites can be filled by a wider range of cations, and on the basis of RNA cross-linking experiments described below, we consider these to be "structure" sites. Precedence for a structural role for these cations comes from tRNA; Mg^{2+}, Mn^{2+}, and Ca^{2+} are the most effective divalent cations in renaturation of tRNA and in stabilization

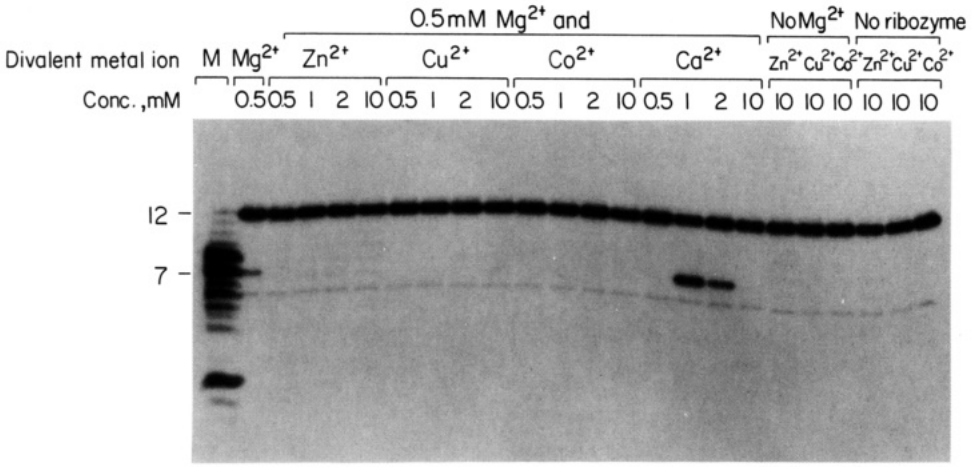

FIGURE 4: Comparison of the effects of different divalent cations on endoribonuclease activity in the presence of 0.5 mM $MgCl_2$. Each 10-μL reaction contained 50 mM Tris-HCl, pH 7.5, 0.5 mM guanosine, 0.2 μM ribozyme, and 2 μM substrate RNA. Reaction time was 20 min, and the temperature was 50 °C. M is a mungbean nuclease digest of substrate RNA.

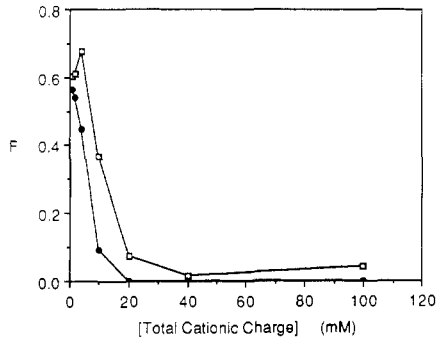

FIGURE 5: Effects of divalent vs monovalent cation on the endoribonuclease reaction at 1 mM $MgCl_2$. Extent of reaction, *F*, as a function of varying concentrations of $CaCl_2$ (□) and NaCl (●). Each 20-μL reaction contained 0.2 μM ribozyme, 2 μM substrate RNA, 50 mM Tris-HCl, pH 7.5, and 0.5 mM guanosine and was incubated at 50 °C for 20 min.

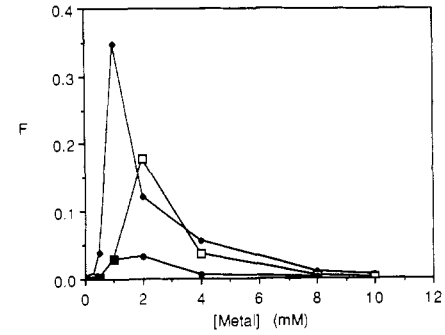

FIGURE 6: Effects of alkaline earth metals, $CaCl_2$ (♦), $SrCl_2$ (□), and $BaCl_2$ (●), on the rate of cleavage of substrate RNA by L - 21 *ScaI* ribozyme in the presence of 0.5 mM $MgCl_2$. Standard conditions were used as described in the legend to Figure 5.

of tRNA against thermal denaturation (Fresco et al., 1966).

Yeast $tRNA^{Phe}$ has at least four strong, site-specific Mg^{2+} binding sites (Holbrook et al., 1977; Jack et al., 1977). It seems reasonable that a folded RNA the size of the IVS (5 times tRNA in size) might have at least 20 such sites. In addition, tRNAs contain 20–40 weak Mg^{2+} or Mn^{2+} binding sites (Danchin, 1972; Stein & Crothers, 1976; Bina-Stein & Stein, 1976), and one would expect such sites in any polynucleotide. Thus, we expect that the *Tetrahymena* IVS RNA has multiple class 2 structure sites, and perhaps multiple class 1 sites as well.

The relative preference of the two classes of sites for different metal ions can be gleaned by a more detailed analysis of the data. The relative ability of Mg^{2+} and Mn^{2+} to act in class 1 or activity sites is best determined in the presence of 1 mM Ca^{2+}, which alleviates the structure requirement (Figure 7B). In this figure, it is seen that Mn^{2+} always gives more activity than the same concentration of Mg^{2+}, or, using an abbreviated notation, $Mn^{2+} > Mg^{2+}$. Similarly, in Table I, comparing rows 3 and 7 or comparing rows 8 and 10 confirms that $Mn^{2+} > Mg^{2+}$. When the concentration of Ca^{2+} is reduced to 0.5 mM (rows 2 and 6), $Mn^{2+} \simeq Mg^{2+}$, and in the absence of Ca^{2+} (rows 1 and 5), $Mg^{2+} > Mn^{2+}$; this switchover is expected if $Mg^{2+} > Mn^{2+}$ in class 2 structure sites. That is, when there is no longer enough Ca^{2+} to fill the structure sites, the data become more and more influenced by the relative abilities of Mg^{2+} and Mn^{2+} to act in structure sites.

Having concluded that class 1 sites prefer Mn^{2+} over Mg^{2+}, it would be useful to be able to distinguish between binding affinity and the activity of the metal ion once it occupies the site. One would normally use the reaction at subsaturating concentrations to evaluate affinity, and reaction at high concentrations to evaluate the activity of the metal ion once bound. In the present case, such analysis is complicated because the reaction does not approach a limiting velocity but is progressively inhibited by metal ion concentration >2 mM. Such inhibition might reflect stabilization of inactive conformers of the RNA (Tanner & Cech, 1985) or increasing the rigidity of the structure such that it has difficult undergoing a conformational transition required for catalysis (Zaug et al., 1988).

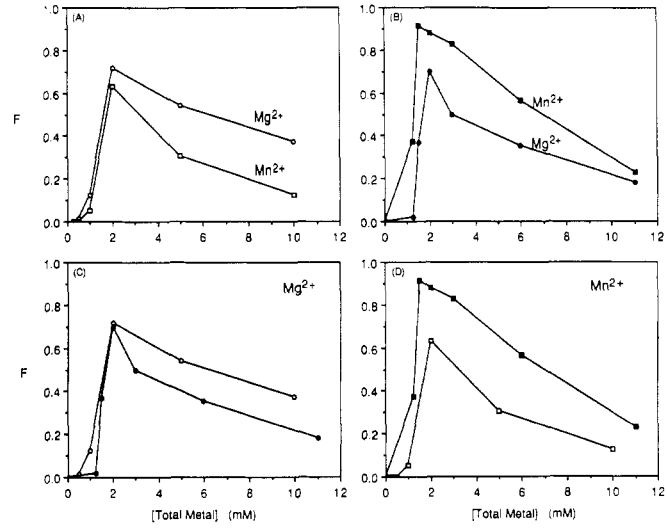

FIGURE 7: Comparison of $[MgCl_2]$ and $[MnCl_2]$ in the endoribonuclease assay with and without $CaCl_2$. (A) Extent of reaction, *F*, as a function of $[MgCl_2]$ (○) and $[MnCl_2]$ (□) without $CaCl_2$. (B) $[MgCl_2]$ (●) compared to $[MnCl_2]$ (■) in the presence of 1 mM $CaCl_2$. [Total metal] includes $CaCl_2$ concentration. (C) Comparison of $[MgCl_2]$ with 1 mM $CaCl_2$ (●) and without (○). (D) Comparison of $[MnCl_2]$ with 1 mM $CaCl_2$ (■) and without (□). All reactions were done under standard conditions.

Cations located at activity sites might have more specific structural roles than those of ions located at class 2 sites. There is ample precedent for different divalent cations forming complexes of different geometry and stability. For example, in complexes with EDTA, Mg^{2+} can be 6- or 7-coordinate, Mn^{2+} is 7-coordinate, and Ca^{2+} is 8-coordinate (Stezowski et al., 1973; Barnett & Uchtman, 1979), and stabilities of these complexes extend over a $>10^5$-fold range. Ca^{2+} has an ionic radius of 0.99 Å, Mn^{2+} is 0.80 Å, and Mg^{2+} is 0.66 Å (Weast, 1987). There could easily be sites in the RNA where Ca^{2+} would not fit at all or would form a complex of incorrect geometry.

Alternatively or in addition, cations located at activity sites might be directly involved in active-site chemistry. Possible roles for metal ions at the active site include stabilization of oxyanions, deprotonation of the 3′-hydroxyl of the incoming guanosine by $M^{2+}(OH^-)$, protonation of the leaving group by $M^{2+}(H_2O)$, and binding to the phosphate that undergoes cleavage, thereby increasing its electrophilicity and stabilizing the transition state (Cech & Bass, 1986; Cech, 1987; Brown et al., 1983; Haydock & Allen, 1985). Sugimoto et al. (1988), studying the $[Mg^{2+}]$ dependence of reverse cyclization of the *Tetrahymena* intron with mixed ribonucleic acid–deoxyribonucleic acid substrates, have concluded that there is a Mg^{2+} ion at the active site for that reaction.

We now consider the class 2 or structure sites. In the absence of Ca^{2+} (Figure 7A), $Mg^{2+} > Mn^{2+}$ despite the greater activity of Mn^{2+} in class 1 sites; thus, $Mg^{2+} > Mn^{2+}$ in class 2 sites. Mixtures of Mn^{2+} plus Ca^{2+} (Figure 7D) always have higher activity than the same total concentration of Mn^{2+} by itself, so $Ca^{2+} > Mn^{2+}$ in class 2 sites. This is confirmed by

comparison of rows 5 and 7 of Table I. Finally, mixtures of Mg^{2+} plus Ca^{2+} (Figure 7C) always have activity less than or equal to the same total concentration of Mg^{2+} by itself, so $Mg^{2+} \geq Ca^{2+}$ in class 2 sites. Combining this information, we conclude that $Mg^{2+} \geq Ca^{2+} > Mn^{2+}$ in class 2 "structure" sites. Furthermore, from Figure 6, it is clear that $Ca^{2+} > Sr^{2+} > Ba^{2+}$.

The virtually complete inhibition of the low [Mg^{2+}] reaction by 10 mM Ca^{2+} (Figures 2 and 4) suggests that Ca^{2+} can compete with Mg^{2+} for class 1 activity sites, but when it fills such sites, it does not potentiate the reaction. Even Na^+ can inhibit the reaction, presumably by causing Mg^{2+} to be displaced (Figure 5). Such effects may represent decreased binding of Mg^{2+} at higher ionic strength rather than specific competition by Ca^{2+} or Na^+. Inhibition is reversible by raising the Mg^{2+} concentration (data not shown). It has been proposed that Na^+ can displace Mg^{2+} even from some strong Mg^{2+} binding sites in tRNA (Jack et al., 1977).

X-ray crystallography of tRNA provides a picture of how divalent cations might stabilize the structure of the intron RNA. Aquo-Mg^{2+} complexes extensively hydrogen bonded to tRNA or Mg^{2+} coordinated directly to backbone phosphates can stabilize sharp turns of the polynucleotide backbone, stabilize hairpin loops, and link two single-stranded regions (Holbrook et al., 1977; Jack et al., 1977). Mn^{2+} has the additional ability to bind to N-7 of guanine (Anderson et al., 1971; Jack et al., 1977), so it is not surprising that it forms a somewhat different coordination complex in tRNA and presumably in the intron RNA. Willick et al. (1973) have suggested that Ca^{2+} can substitute for Mg^{2+} with no change in the conformation of tRNA whereas Mn^{2+} produces a conformational change detectable by circular dichroism. We draw similar conclusions about the relative abilities of these three cations to stabilize the ribozyme.

Recent analysis of a long-range UV cross-link in the *Tetrahymena* ribozyme further justifies the conclusion that Ca^{2+} as well as Mg^{2+} can stabilize a specific structure of the intron. The cross-link does not form in EDTA but does form in 1.5 mM Mg^{2+}, in 1.5 mM Ca^{2+}, or in 1.0 mM Ca^{2+} + 0.5 mM Mg^{2+} (W. Downs and T. R. Cech, unpublished results).

Metal Ion Requirements for Other Reactions of the Tetrahymena Ribozyme. The data presented here concern only the guanosine addition reaction catalyzed by the L - 21 *Sca*I RNA. Other reactions of the *Tetrahymena* intron have a considerably higher [Mg^{2+}] optimum (10–50 mM). These include cyclization of the intron (Cech et al., 1981), an intermolecular version of cyclization (Tanner & Cech, 1987), reverse cyclization (Sullivan & Cech, 1985; Sugimoto et al., 1988), and transesterification reactions between dinucleotides (Kay & Inoue, 1987). Even guanosine addition at the 5′ splice site, a reaction equivalent to that studied here, can have a high [Mg^{2+}] requirement in a system where the ribozyme is assembled from two fragments (Szostak, 1986). One reasonable interpretation is that all the reactions listed above involve weak interactions and therefore have a structure requirement in addition to the basic structure requirement for the endoribonuclease reaction. In evaluating any of the activity vs metal ion concentration graphs, it is useful to consider that they are dominated by the most weakly bound metal ion that is required for activity.

Comparison to Other RNA Enzymes. The RNA subunit of ribonuclease P from *E. coli* or *Bacillus subtilis* can cleave the 5′ leader sequence from tRNA precursors in vitro in the absence of protein (Guerrier-Takada et al., 1983). The RNase P RNAs have [Mg^{2+}] optima of ≥100 mM, much higher than that of the *Tetrahymena* ribozyme (Gardiner et al., 1985; Guerrier-Takada et al., 1986). Nevertheless, a detailed analysis led Guerrier-Takada et al. (1986) to propose two classes of metal ion binding sites analogous to those we propose for the *Tetrahymena* ribozyme. They concluded that Mg^{2+}, Ca^{2+}, Sr^{2+}, and to a lesser extent Mn^{2+} served a required structural role, whereas Mg^{2+} or Mn^{2+} was necessary for activity. These authors further concluded that the activity site was at the catalytic center of RNase P, a proposal that we consider to be chemically reasonable but not proven by the data for either RNase P or the *Tetrahymena* ribozyme.

Some information is available concerning metal ion requirements for self-splicing of group II introns, a transesterification reaction which involves formation of a branched lariat RNA. Self-splicing is optimal in ~10 mM Mg^{2+} plus 1–5 mM spermidine (Peebles et al., 1986; Van der Veen et al., 1986). Thus, once again the cations might be serving to promote both structure (spermidine) and activity (Mg^{2+}).

Another class of ribozymes are those derived from the plant infectious agents, virusoids and satellite RNAs. Unlike the self-splicing RNAs and RNase P, some of these RNAs undergo significant reaction (self-cleavage) in the absence of metal ions, but in all cases, their rate is enhanced by Mg^{2+} and other divalent cations (Prody et al., 1986; Hutchins et al., 1986; Forster & Symons, 1987). When the self-cleaving plant RNAs are divided into two components, one RNA fragment serving as enzyme and one as substrate, there is then an absolute metal ion requirement that can be met by Mg^{2+}, Mn^{2+}, or Ca^{2+} (Uhlenbeck, 1987).

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Acid Dissociation Constant and Apparent Nucleophilicity of Lysine-501 of the α-Polypeptide of Sodium and Potassium Ion Activated Adenosinetriphosphatase†

Kai-yuan Xu

Department of Chemistry, University of California at San Diego, La Jolla, California 92093

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ABSTRACT: A combination of competitive labeling with [^{3}H]acetic anhydride [Kaplan, H., Stevenson, K. J., & Hartley, B. S. (1971) *Biochem. J. 124*, 289-299] and immunoaffinity chromatography is described that permits the assignment of the acid dissociation constant and the absolute nucleophilicity of individual lysines in a native enzyme. The acid dissociation constant of lysine-501 of the α-polypeptide in native (Na^+ + K^+)-ATPase was determined. This lysine had a normal pK_a of 10.4. The rate constant for the reaction of the free base of lysine-501 with acetic anhydride at 10 °C is 400 M^{-1} s^{-1}. This value is only 30% that for a fully accessible lysine in a protein. The lower than normal apparent nucleophilicity suggests that lysine-501 is hindered from reacting with its intrinsic nucleophilicity by the tertiary structure of the enzyme and is consistent with its location within a pocket that forms the active site upon the surface of the native protein.

Sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase][1] (Skou, 1957) is the enzyme responsible for the coupled, active transport of sodium and potassium across the plasma membranes of all animal cells (Kyte, 1981). The enzyme is composed of one α-subunit and one β-subunit (Craig, 1982). The α-subunit, composed from a polypeptide with a length of 1016 amino acids (Shull et al., 1985; Ovchinnikov et al., 1986), is responsible for catalysis (Kyte, 1981). The β-subunit is a glycoprotein (Kyte, 1972) composed from

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[1] Abbreviations: (Na^+ + K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); HPLC, high-pressure liquid chromatography; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate.

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