Role of Divalent Metal Ions in the Hammerhead RNA Cleavage Reaction†

SueAnn C. Dahm and Olke C. Uhlenbeck*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

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ABSTRACT: A hammerhead self-cleaving domain composed of two oligoribonucleotides was used to study the role of divalent metal ions in the cleavage reaction. Cleavage rates were measured as a function of $MgCl_2$, $MnCl_2$, and $CaCl_2$ concentration in the absence or presence of spermine. In the presence of spermine, the rate vs metal ion concentration curves are broader, and lower concentrations of divalent ions are necessary for catalytic activity. This suggests that spermine can promote proper folding of the hammerhead and one or more divalent ions are required for the reaction. Six additional divalent ions were tested for their ability to support hammerhead cleavage. In the absence of spermine, rapid cleavage was observed with Co^{2+} while very slow cleavage occurred with Sr^{2+} and Ba^{2+}. No detectable specific cleavage was observed with Cd^{2+}, Zn^{2+}, or Pb^{2+}. However, in the presence of 0.5 mM spermine, rapid cleavage was observed with Zn^{2+} and Cd^{2+}, and the rate with Sr^{2+} was increased, indicating that while these three ions could not promote proper folding of the hammerhead they were able to stimulate cleavage. These results suggest certain divalent ions either participate directly in the cleavage mechanism or are specifically involved in stabilizing the tertiary structure of the hammerhead. Additionally, an altered divalent metal ion specificity was observed when a unique phosphorothioate linkage was inserted at the cleavage site. The substitution of a sulfur for a nonbridging oxygen atom substantially reduced the affinity of an important Mg^{2+} ion necessary for efficient cleavage. In contrast, the reaction proceeds normally with Mn^{2+}, presumably due to its ability to coordinate with both oxygen and sulfur. This suggests that a divalent ion coordinates directly to the phosphate at the cleavage site.

As part of their rolling circle replication mechanisms (Branch et al., 1981), the genomes of several plant satellite RNAs are capable of autolytic cleavage at a unique location (Hutchins et al., 1986; Prody et al., 1986). Cleavage is the result of a transesterification reaction and generates 2′,3′-cyclic phosphate and 5′-hydroxyl termini. For the genomic strands of satellite tobacco ringspot virus (Buzayan et al., 1986a,b) and lucerne transient streak virus (Forster & Symons, 1987), fewer than 60 contiguous nucleotides of the RNA genome are needed for cleavage. A consensus secondary structure for self-cleavage termed the hammerhead has been proposed (Keese & Symons, 1987). The hammerhead domain can be assembled by combining two or three oligoribonucleotides in several different ways (Uhlenbeck, 1987; Haseloff & Gerlach, 1988; Koizumi et al., 1988; Ruffner et al., 1989; Jeffries & Symons, 1989), thereby defining ribozyme and substrate oligoribonucleotides. In the presence of an excess of substrate oligoribonucleotide, catalytic cleavage of the substrate can be observed (Uhlenbeck, 1987; Haselhoff & Gerlach, 1988; Jeffries & Symons, 1989). Because of its small size and relatively simple secondary structure, the hammerhead is an attractive system to study the structure and mechanism of an RNA enzyme.

This paper focuses on the role of divalent metal ions in the hammerhead self-cleavage reaction. Although Mg^{2+} is usually included in cleavage reactions, examples of cleavage in the absence of added divalent metal ions have been reported (Prody et al., 1986; Forster et al., 1987). Divalent ions are effective counterions to neutralize negative charges on phosphates. In addition, high-affinity divalent ion binding sites involving hydrogen bonding to amine and carbonyl groups have been found in tRNAs (Holbrook et al., 1977; Jack et al., 1977). Since the hammerhead has secondary and probably tertiary structure as well, both such classes of divalent ion binding sites may exist. Further, it is possible that divalent metal ions could be involved in the mechanism of the hammerhead cleavage reaction. For example, several divalent metals are capable of promoting cleavage of yeast $tRNA^{Phe}$ at a unique site (Werner et al., 1976; Rordorf & Kearns, 1976; Behlen et al., 1990).

A hammerhead self-cleaving domain composed of two oligoribonucleotides was used to study the role of divalent metal ions in the cleavage reaction. This hammerhead configuration has the general features of a small target substrate and a large ribozyme that is similar to those used to cleave chloramphenicol acetyltransferase mRNA (Haseloff & Gerlach, 1988). The substrate oligoribonucleotide was also designed such that a unique phosphorothioate could be placed at the cleavage site to permit the unambiguous analysis of the effect of this linkage on the cleavage rate. During the course of these studies, we found that the phosphorothioate substrate showed an unusual divalent metal ion specificity.

Materials and Methods

RNA Synthesis. RNA was synthesized by in vitro transcription with T7 RNA polymerase using synthetic DNA templates (Milligan et al., 1987). Transcription reactions contained 40 mM Tris-HCl (pH 8.1 at 37 °C), 6 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 0.01% Triton X-100, 1 mM NTPs, 100–300 nM DNA template, and 0.1–0.25 mg/mL T7 RNA polymerase. Reactions were incubated for 1–2 h at 37 °C. Phosphorothioate RNA was prepared by replacing ATP with the S_p isomer of adenosine 5′-*O*-(1-thiotriphosphate) (gift from Dr. F. Eckstein). Incorporation of phosphorothioates using T7 RNA polymerase results in inversion of configuration with an RNA transcript containing

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the R_p isomer (Griffiths et al., 1987). For [α-^{32}P]CTP-labeled transcripts, the total concentration of CTP was lowered to 100 nM, and 150–200 μCi of [α-^{32}P]CTP (3000 Ci/mmol) was added to a 50-μL transcription reaction.

Oligoribonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis. Transcription products were located by either autoradiography or UV shadowing. Most transcription reactions produced numerous shorter length RNAs as well as the full-length product band plus a transcript one nucleotide longer. For ribozyme RNA isolation, the product band was crushed and soaked overnight in 2 volumes of a buffer containing 0.5 M NaOAc, 0.1 M Tris (pH 8.0), and 1 mM EDTA. The extracted RNA was concentrated by ethanol precipitation and resuspended in water. Substrate RNAs were isolated by a rapid crush and soak procedure and stored in dithiothreitol to minimize degradation of thio-substituted RNA (Milligan & Uhlenbeck, 1989). To verify that the substrate and ribozyme transcripts were the correct lengths, the transcripts were 3′ end labeled with [5′-^{32}P]pCp and RNA ligase and subjected to total digestion with ribonuclease T_2 (England & Uhlenbeck, 1978). The digestion product nucleosides were separated and identified by 2D thin-layer chromatography (Kochino et al., 1980).

Cleavage Reactions. To measure the cleavage rate in the absence of added divalent ions, all reaction components including the RNA were treated with diphenylthiocarbazone to remove any potentially contaminating divalent metal ions (Holmquist, 1988). Stock solutions of water, Tris buffer, EDTA, spermine, and both ribozyme and substrate RNA were individually extracted with an equal volume of 15 μM diphenylthiocarbazone in water-saturated chloroform. The mixtures were vortexed for 30 s and allowed to stand at room temperature for 5 min prior to centrifugation to recover the individual aqueous phases. Control experiments showed that the residual amount of chloroform in the reaction did not alter the cleavage rate.

Cleavage reactions contained 50 mM Tris-HCl (pH 7.5), 50 nM substrate oligoribonucleotide, 100 nM enzyme oligoribonucleotide, and varying concentrations of divalent ions; 0.5 mM spermine was also used in some reactions. All reactions were performed at 25 °C. The reaction protocol involved combining substrate and buffer in one tube which was heated to 90 °C for 1 min. After the tube was slowly cooled to 25 °C, spermine and/or divalent ions were added. An aliquot of the ribozyme in water was heated to 90 °C for 1 min and slowly cooled to 25 °C. Reactions were initiated by the addition of ribozyme, and aliquots were taken at appropriate intervals. Reactions were terminated by the addition of an equal volume of 7 M urea, 50 mM EDTA, 0.02% bromphenol blue, and 0.02% xylene cyanol. Reaction products were separated by denaturing 20% polyacrylamide gel electrophoresis and located by autoradiography. The two expected cleavage products, which comigrated as a single band under these gel conditions, were excised from the gel along with the labeled substrate. The radioactivity in each band was determined by liquid scintillation counting, and the fraction of cleavage was calculated at each time point. Each cleavage rate was calculated from at least seven time points. Cleavage rate versus metal ion concentration curves were best fit to sigmoid curves with adjustable cooperativity parameters.

Results and Discussion

Hammerhead Cleavage with Divalent Ions. The hammerhead self-cleaving domain used in these studies is shown in Figure 1A. It consists of a 33-residue ribozyme and a 13-residue substrate. The expected site of cleavage is indicated by an arrow. The time course of a typical cleavage reaction that contained 100 nM ribozyme and 50 mM substrate in 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ at 25 °C is shown in Figure 1B. The apparent rate constant for the reaction was 0.15 min^{-1}. Under these conditions, this hammerhead cleaves somewhat slower than two of the four previously reported hammerheads of similar configuration (Ruffner et al., 1989).

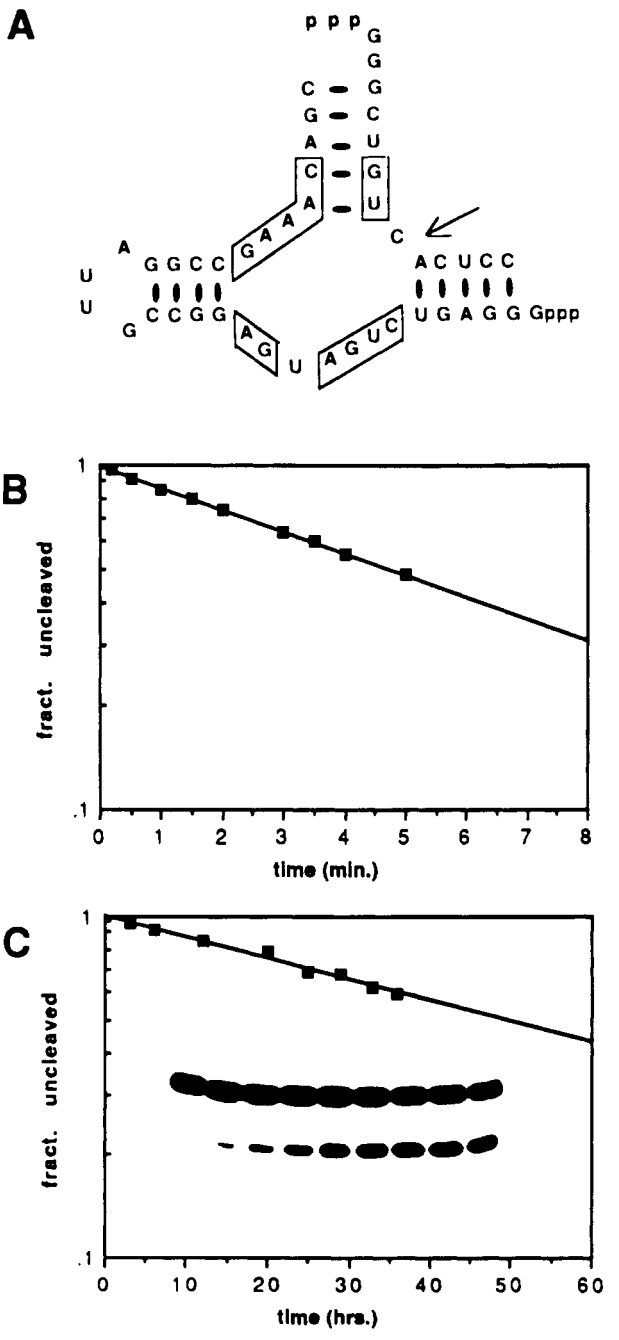

FIGURE 1: (A) Hammerhead self-cleaving domain with conserved nucleotides boxed. The arrow indicates the cleavage site. (B) Time course of cleavage in 10 mM $MgCl_2$ at 25 °C. (C) Time course of cleavage in 0.5 mM spermine + 1 mM EDTA at 25 °C. (Insert) Audioradiogram of a polyacrylamide gel showing cleavage kinetics.

The initial rates of cleavage in 50 mM Tris-HCl, pH 7.5, and varying concentrations of $MgCl_2$, $MnCl_2$, and $CaCl_2$ are shown in Figure 2A. In the absence of the added divalent ions, no cleavage was observed even after incubation for as long as 24 h. For each divalent ion tested, the reaction rate increases rapidly over a relatively narrow range of concentration and then reaches a plateau. Since the curves are much steeper than would be predicted for a simple binding equi-

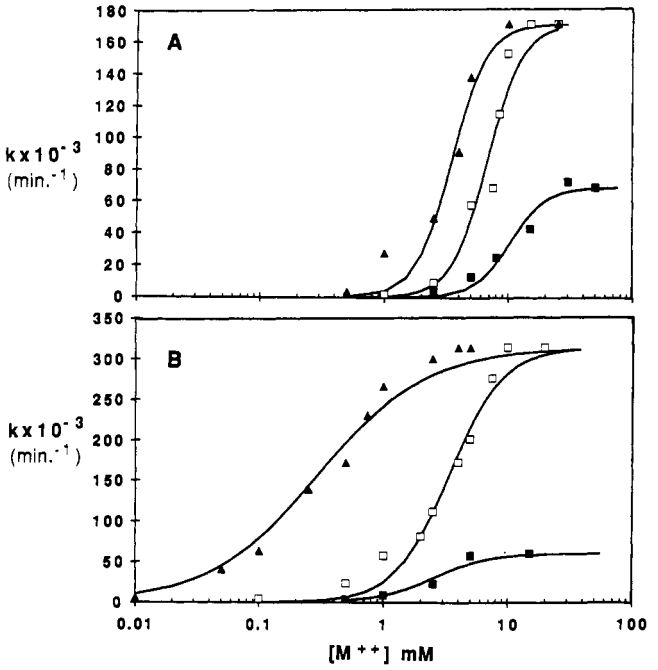

FIGURE 2: (A) Hammerhead cleavage rates as a function of divalent ion concentrations: (▲) Mn^{2+}; (□) Mg^{2+}; (■) Ca^{2+}. (B) Hammerhead cleavage rates as a function of divalent ion concentrations in the presence of 0.5 mM spermine.

librium (cooperativity values of 2–3), cooperative ion binding appears required for cleavage. Cooperative divalent ion binding to tRNA (Cohn et al., 1969; Danchin & Gueron, 1970) is believed to reflect a large cooperative rearrangement of the tRNA chain as the divalent ions are added in low-salt buffer (Stein & Crothers, 1976). The compact, native tRNA binds divalent ions more strongly than the partially unfolded forms extended by electrostatic forces (Cole et al., 1972). Similarly, the two RNAs that form the hammerhead are not expected to associate in the buffer due to the substantial charge repulsion that must occur between the strands of the two RNA helices as well as within the folded core of the hammerhead. Indeed, the midpoint of the activity curves in Figure 2A corresponds closely with the reported affinity of divalent ions to RNA (Cohn et al., 1969; Romer & Häch, 1975). The different activities for Mg^{2+}, Mn^{2+}, and Ca^{2+} may reflect their differing abilities either to function as counterions to promote assembly of the hammerhead, or to specificially stabilize the tertiary structure, or to participate in the mechanism of the cleavage reaction.

In an attempt to promote assembly of the hammerhead in the absence of divalent ions, several monovalent salts and polyamines were tested for their ability to stimulate the cleavage rate at a subsaturating magnesium ion concentration. While stimulation was often observed, spermine at an optimal concentration of 0.5 mM was found to be the most effective. Under these conditions, charge repulsion is greatly reduced due to the presence of the alternate cations, and thus divalent cation binding affinities would be expected to be weaker. As shown in Figure 2B, in each case the presence of spermine not only substantially broadens the rate versus metal ion concentration curves but also shifts the midpoint to a lower concentration. In addition, the rates are nearly doubled at saturating Mg^{2+} and Mn^{2+} (but not Ca^{2+}) concentrations compared to the rates measured in the absence of spermine. The fact that divalent ions remain required for cleavage under conditions where charge repulsion is greatly reduced suggests that certain divalent ions are either directly involved in the cleavage mechanism or participate in the folding in a highly specific manner. The much lower observed cooperativity (cooperativity values of 1–2) compared to the curves in the absence of spermine indicates that a cooperative rearrangement of the hammerhead structure does not occur. Although the number of divalent ions that are still required for cleavage cannot be determined from the experiment, only a very few could be involved. Analogous to certain metal ion binding sites in tRNA (Cohn et al., 1969; Jack et al., 1977; Teeter et al., 1981), the hammerhead may contain a metal ion binding pocket where the metal ion not only can form ionic interactions but also can coordinate via hydrogen bonds from the hydrated metal ion with nucleotide or backbone functional groups.

Table I: Cleavage Rates with Divalent Ions in 50 mM Tris, pH 7.5

metal	$k_{obs} \times 10^{-3}$ (min^{-1}) 10 mM M^{2+}	0.5 mM spermine, 2 mM M^{2+}	ionic radius (Å)[c]
Mg	154	82	0.66
Co	139	58	0.72
Zn	<0.24	46[a]	0.74
Mn	173	277	0.80
Cd	<0.24	277	0.97
Ca	30	20	0.99
Sr	1.2	11	1.12
Pb	<0.24	<1[b]	1.20
Ba	1.3	1.1	1.34
none		0.24	

[a] 0.5 mM Zn^{2+} was used; higher concentrations inhibited the reaction. [b] 0.2 mM Pb^{2+} was used to minimize degradation. [c] Weast (1987).

Six additional divalent ions were tested for their ability to support hammerhead cleavage (Table I). By measurement of rates either without or with spermine, the ability of the different ions to promote folding and cleavage or just cleavage could be evaluated. Similar to Mg^{2+} and Mn^{2+}, rapid cleavage was observed in 10 mM Co^{2+} at pH 7.5. Very slow rates of cleavage were observed with Sr^{2+} and Ba^{2+}, and no detectable specific cleavage was observed with Cd^{2+}, Zn^{2+}, and Pb^{2+} although some ribozyme-independent nonspecific cleavage of the substrate was observed in the latter case as would be expected (Britten, 1962; Farkas, 1968). When 0.5 mM spermine was included to stabilize the structure, rapid cleavage was now observed with both Zn^{2+} and Cd^{2+}, suggesting that while these ions could not promote proper folding they were able to stimulate cleavage. A substantial increase in the rate with Sr^{2+} was also observed, while the rates with Ba^{2+} and Pb^{2+} were not improved. When spermine is present, there is a clear correlation between the radius of the ion and the ability to stimulate the reaction. All the ions with ionic radius less than 1 Å were active while the larger ions were not. This suggests that the divalent ion binding site must have very specific size requirements.

The large number of cations which are able to stimulate hammerhead cleavage contrasts with metal ion specificity studies with other catalytic RNAs. Two classes of sites have been defined for the endoribonuclease activity of the *Tetrahymena* self-splicing intron (Grosshans & Cech, 1989). Class I sites can bind either Mg^{2+} or Mn^{2+}, which appear to be involved in active-site chemistry, while class II sites bind several additional metals that are presumably involved in RNA folding. The metal ion specificity of the catalytic RNA subunit of *Escherichia coli* RNase P suggest a similar distinction in the metal ions needed for the reaction (Guerrier-Takada et al., 1986). Again, only Mg^{2+} or Mn^{2+} is capable of promoting cleavage although RNA folding can be achieved with several additional metals. As would be expected, the ions which

successfully stabilize the structure of the three catalytic RNAs are quite similar. With the exception of Zn^{2+} and Cd^{2+}, all the smaller divalent ions can promote folding. Surprisingly, Co^{2+} can stabilize hammerhead folding, but not the other two ribozymes. The more restrictive metal ion dependence for catalysis observed for RNase P and the *Tetrahymena* intron presumably reflects the mechanistic differences of these reactions.

It has been noted (Brown et al., 1985; Cech, 1987; Behlen et al., 1990) that the nonphysiological specific cleavage of yeast $tRNA^{Phe}$ at position 17 by metal ions resembles the hammerhead reaction in the sense that both give a 2′,3′-cyclic phosphate and 5′-hydroxyl termini as cleavage products. Specific $tRNA^{Phe}$ cleavage is believed to result from a specifically bound metal hydroxyl [$M(OH)^+$] that can abstract the proton of the 2′-hydroxyl of ribose 17 and thereby facilitate nucleophilic attack on the phosphodiester bond (Brown et al., 1985; Behlen et al., 1990). In addition to similar cleavage termini, we show here that the two reactions are also similar in the sense that a variety of divalent ions are active in the reaction (Werner et al., 1976; Rordorf & Kearns, 1976). $tRNA^{Phe}$ cleavage, however, is much more active with metals with p*K*'s around neutrality than the hammerhead. For example, Pb^{2+} with a p*K* of 7.5 is the most active ion in the cleavage of $tRNA^{Phe}$ but virtually inactive with the hammerhead. However, this lack of activity may be due in part to the comparatively large ionic radius of Pb^{2+} which makes it unable to fit into the metal ion pocket in the hammerhead. Indeed, this differing metal ion specificity does not rule out the metal hydroxyl catalyzed mechanism for the hammerhead reaction since divalent metals can vary substantially in both their affinity and their position when interacting at specific sites in RNA. For example, in crystallographic studies of different metal ions binding to specific sites in yeast $tRNA^{Phe}$ (Jack et al., 1977; Rubin et al., 1983; Rubin & Sundaralingam, 1983), the position of different divalents in a pocket can vary by as much as 2 Å, presumably because of the varying sizes and differential preferences of the divalent ions for the coordinating ligands. Indeed, one Mg^{2+} binding site, Mg (3), will bind Mn^{2+}, Zn^{2+}, and Pb^{2+} although the exact position and coordination ligands differ.

It is interesting that in the presence of spermine or monovalent ions plus 1 mM EDTA, low rates of cleavage can be observed in the absence of added divalent ions. While it was not possible to rigorously establish the complete absence of divalent ions in the reaction, the RNA was purified under denaturing conditions and in the presence of chelators. In addition, the RNAs and buffer were pretreated with the strong chelator diphenylthiocarbazone. The kinetics of cleavage in 50 mM Tris-HCl, pH 7.5, 0.5 mM spermine, and 1 mM EDTA are shown in Figure 1C. Although the cleavage rate is slow, the correct cleavage products are obtained, and no cleavage is observed when the ribozyme is omitted. The observed rate of 2.4×10^{-4} min^{-1} is approximately 1300-fold slower than the same reaction with 10 mM $MgCl_2$ plus spermine added. Increasing the ribozyme concentration 70-fold and using a trace amount of labeled substrate did not alter the reaction rate, indicating that saturation of substrate was achieved in the spermine buffer. Similar rates of cleavage were observed when EDTA was replaced by the same concentration of the chelators EGTA and CDTA. Thus, it is possible that a slow rate of cleavage can occur in the absence of divalent ion provided that the structure is stabilized in some other manner.

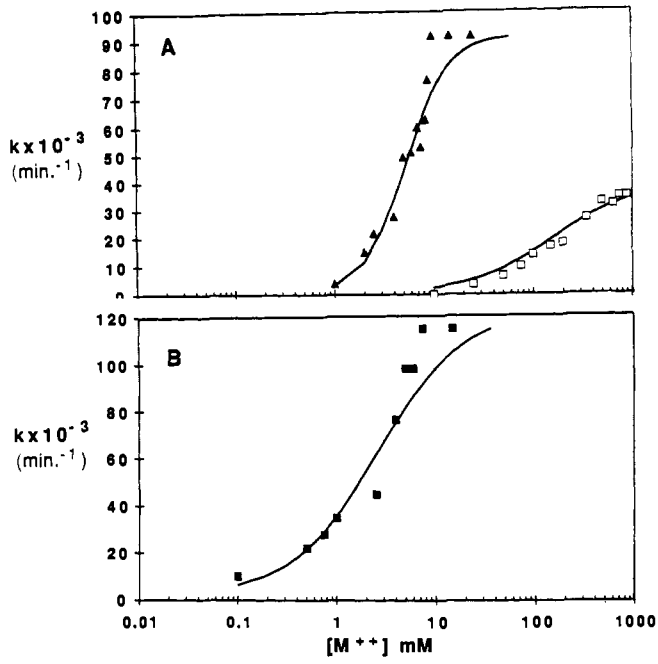

FIGURE 3: (A) Thio-substituted hammerhead cleavage rates as a function of divalent ion concentrations: (▲) Mn^{2+}; (□) Mg^{2+}. (B) Thio-substituted hammerhead cleavage rates as a function of $MnCl_2$ concentration in the presence of 10 mM $MgCl_2$.

A Hammerhead with a Unique Phosphorothioate at the Cleavage Site. The sequence of the substrate strand was designed such that when ATPαS is used in place of ATP in the transcription reaction, an RNA with a unique phosphorothioate linkage at the cleavage site is obtained. While the effects of phosphorothioates on hammerhead cleavage have previously been studied (Buzayan et al., 1988), the data are complicated by the fact that they contained multiple phosphorothioate linkages, and it has recently been shown that phosphorothioates at three sites other than the cleavage site can inhibit cleavage (Ruffner & Uhlenbeck, 1990). The initial rates of cleavage of the singly substituted hammerhead as a function of Mn^{2+} and Mg^{2+} concentrations are shown in Figure 3A. The curve for Mn^{2+} closely resembles the data for the normal substrate (Figure 2A) except that a 2-fold lower rate is observed at saturating Mn^{2+} concentration. In contrast, the curve for Mg^{2+} is substantially broader and shifted to a 100-fold higher concentration than is needed for the normal substrate. These data also suggest that the hammerhead contains a "special" metal ion binding site that is needed for cleavage. While both ions can bind to this site in the normal hammerhead, the replacement of a single oxygen by a sulfur does not greatly alter Mn^{2+} binding, but substantially reduces the affinity of Mg^{2+}. Thus, although the thio-substituted hammerhead can assemble in 10 mM Mg^{2+}, much higher concentrations of Mg^{2+} are required to bind to the specific site with the broad activity curve reflecting the titration of that site. This explanation is consistent with the observation that when 10 mM $MgCl_2$ is present, lower concentrations of Mn^{2+} can be used to cleave the thio-substituted substrate (Figure 3B). The $MgCl_2$ acts analogously to spermine in Figure 2B in stabilizing the structure of the hammerhead so the activity curve now reflects introduction of Mn^{2+} into a site important for efficient cleavage.

At saturating concentrations of Mn^{2+} or Mg^{2+}, the cleavage rate of the thio-substituted hammerhead is 2–5 times lower than its unsubstituted counterpart. This rate difference, called the thio effect, is likely to reflect the chemistry of the reaction which is rate-limiting for this hammerhead under saturating

conditions (Fedor & Uhlenbeck, 1990). While thio effects of this magnitude have been found for certain ribonucleases (Burgers & Eckstein, 1979) and phosphate diester model compounds (Herschlag et al., 1991), the analogous nonenzymatic base-catalyzed hydrolysis of RNA shows virtually no thio effect (Burgers & Eckstein, 1979; Herschlag et al., 1991). This difference may be the result of the larger, more electronegative R_p sulfur (Frey & Sammons, 1985) making an unfavorable interaction with some part of the hammerhead in the transition state. Alternatively, the ribozyme may proceed by a different reaction mechanism than the nonenzymatic reaction.

The striking metal ion specificity of the thio effect supports direct coordination of a metal ion with the *pro*-R_p oxygen atom of the phosphate at the hammerhead cleavage site. On the basis of studies measuring the affinity of divalent ions with ATPβS (Jaffe & Cohn, 1979; Pecoraro et al., 1984), Mg^{2+} is coordinated 30000-fold more strongly to oxygen than sulfur while Mn^{2+} coordinates the two atoms more equally. As a result, this thio effect is quite small with Mn^{2+} since it can bind in either case. However, the poor binding of Mg^{2+} to sulfur results in a very large thio effect until high concentrations of Mg^{2+} are reached.

The direct coordination of a divalent ion to the phosphate at the cleavage site could stimulate the reaction in several ways. The observed 120-fold stimulation of the intramolecular transesterification of lactic acid *O*-phenyl phosphate by divalent ions is suggested to be the result of stabilization of either the transition state or the reaction product (Steffens et al., 1975). Thus, the hammerhead could contain an ion pocket that positions two of the six octahedrally arranged coordination sites of a divalent ion so that they stabilize the pentacoordinate transition state by, for example, binding the *pro*-R_p oxygen and the 5′-oxygen of the leaving group. Alternatively, the metal ion coordinated to the *pro*-R_p oxygen could be involved in acid/base catalysis by supplying a hydroxyl ion to deprotonate the attacking 2′-hydroxyl as well as a water molecule to protonate the 5′-leaving group (Haydock & Allen, 1985). While divalent ions stimulate the hammerhead cleavage rate by more than a thousandfold, the apparent rate of cleavage in the absence of divalent ions (10^{-4} min^{-1}) is at least 4 orders of magnitude faster than estimates for the random cleavage of RNA at neutral pH (Shapiro & Vallee, 1989; Cech, 1987). While the remaining rate enhancement may be the result of precise orientation of the attacking 2′-hydroxyl (Mei et al., 1989), the possibility of acid or base catalysis aided by a hydrated metal complex or nucleotide functional groups still remains.

Acknowledgments

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Registry No. Mg, 7439-95-4; Mn, 7439-96-5; Ca, 7440-70-2; spermine, 71-44-3.

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Actinomycin D and 7-Aminoactinomycin D Binding to Single-Stranded DNA

Randy M. Wadkins‡ and Thomas M. Jovin*
Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Postfach 2841, W-3400 Göttingen, Federal Republic of Germany
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ABSTRACT: The potent RNA polymerase inhibitors actinomycin D and 7-aminoactinomycin D are shown to bind to single-stranded DNAs. The binding occurs with particular DNA sequences containing guanine residues and is characterized by hypochromic UV absorption changes similar to those observed in interactions of the drugs with double-stranded duplex DNAs. The most striking feature of the binding is the dramatic (ca. 37-fold) enhancement in fluorescence that occurs when the 7-aminoactinomycin is bound to certain single-stranded DNAs. This fluorescence of the complex is also characterized by a 40-nm hypsochromic shift in the emission spectrum of the drug and an increase in the emission anisotropy relative to the free drug or the drug bound to calf thymus DNA. The fluorescence lifetimes change in the presence of the single-stranded DNA in a manner compatible with the intensity difference. Thus, there is an increase in the fraction of the emission corresponding to a 2-ns lifetime component compared to the predominant ~0.5-ns lifetime of the free drug. The 7-aminoactinomycin D comigrates in polyacrylamide gels with the single-stranded DNAs, and the fluorescence of the bound drug can be visualized by excitation with 540-nm light. The binding interactions are characterized by association constants of 2.0×10^6 to 1.1×10^7 M^{-1}.

Actinomycin D (ACTD)[1] is a compound with a variety of biological properties [for a review, see Waring, (1981)]. It has been used clinically to treat numerous tumors (Farber, 1966; Lewis, 1972). It is also a potent inhibitor of transcription both in vivo (Goldberg & Friedman, 1971; Kersten & Kersten, 1974) and in vitro (Reich, 1964; Wells & Larson, 1970; Straney & Crothers, 1987; White & Phillips, 1988). The transcriptional inhibition is thought to occur through binding of the drug to DNA and blockage of the RNA polymerase during elongation (Mueller & Crothers, 1968; Sentenac et al., 1968; Aivasashvilli & Beabealashvilli, 1983; Straney & Crothers, 1987; White & Phillips, 1988), inasmuch as ACTD has not been observed to bind to RNA (Goldberg & Friedman, 1971; Kersten & Kersten, 1974; Bunte et al., 1980).

The binding of ACTD to DNA is characterized by a general requirement for G/C residues that can be fullfilled by either a ^{5}G–C^3 or ^{5}C–G^3 step. However, the binding to the latter sequence is substantially weaker (Wilson et al., 1986; Chen, 1988; Zhou et al., 1989). Crystal structures of the drug complexed with dG and dGpdC suggest that the structural basis for the G/C requirement is the need for hydrogen bonding between the carboxyl of the threonine residue of the drug and the guanine exocyclic 2-amino protons (Jain & Sobell, 1972; Takusagawa et al., 1982). Additionally, the flanking base pairs at such G–C or C–G steps also greatly influence the binding properties of the drug (Chen, 1988a,b, 1990; White & Phillips, 1988), making it clear that the mechanism of binding of ACTD is complex and cannot be specified by a simple nearest-neighbor requirement.

ACTD also binds to sequences that do not contain either G–C or C–G sites, for example poly(dI) (Wells & Larson, 1970). In addition, binding of the drug has been observed to the synthetic polynucleotides poly[d(GTA)]·poly[d(TAC)], poly[d(GAA)]·poly[d(TTC)], and poly[d(TTG)]·poly[d(CAA)], all of which contain only a single d(G·C) base pair in tracts of A's and T's (Wells & Larson, 1970). Interestingly, no interaction was observed with poly[d(GAT)]·poly[d(ATC)], emphasizing that the sequence of the DNA is the primary

* To whom correspondence should be addressed.
‡ Present address: Department of Biochemical & Clinical Pharmacology, St. Jude Children's Research Hospital, Memphis, TN.

[1] Abbreviations: ACTD, actinomycin D; 7AACTD, 7-aminoactinomycin D; TBE, 90 mM tris(hydroxymethyl)aminomethane, 90 mM boric acid, 2.5 mM ethylenediaminetetraacetic acid, pH 8.3; TBM, 90 mM tris(hydroxymethyl)aminomethane, 90 mM boric acid, 2.5 mM $MgCl_2$, pH 8.3.

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