Mg^{2+}-Dependent Conformational Changes in the Hammerhead Ribozyme†

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ABSTRACT: Conformational changes of the hammerhead ribozyme were examined by fluorescence changes of 2-aminopurine riboside incorporated either in the substrate or in the ribozyme. Fluorescence changes could be observed for both the substituted substrate and ribozyme upon complex formation, indicating a different environment for the 2-aminopurine in the complex. Ribozyme−substrate constructs for cis-cleavage containing 2-aminopurine at various sites were used for the determination of binding constants of Mg^{2+} and Ca^{2+}. Depending upon the site of 2-aminopurine substitutions, the fluorescence intensity upon addition of Mg^{2+} or Ca^{2+} was reduced by 0−50%. The measurements were performed in high ionic strength buffers such that base pairing in the helical regions is expected to be complete. With three of the ribozymes, the dependence of the fluorescence emission as a function of Mg^{2+} concentration could be fitted by single binding processes, whereas for the two remaining ribozymes a second binding process needed to be included. The binding constants range from 7600 M^{-1} down to 12 M^{-1} in 75 mM Tris-HCl (pH 7.5) and indicate the presence of multiple binding sites in the ribozymes with varying degrees of affinity toward the metal ions. Mg^{2+} binding constants determined in the same buffer from the Mg^{2+} dependence of the cleavage rate are of the order of 100 M^{-1}; thus, Mg^{2+} sites directly involved in catalysis are of intermediate affinity. The ribozyme containing 2-aminopurine in loop III demonstrated the highest binding constant whereas the ribozyme with a 2-aminopurine next to a 2′-deoxy-2′-aminocytosine at the cleavage site exhibited only low metal ion affinity. The data obtained for Ca^{2+} are very similar to those found for Mg^{2+}. This approach provides a first set of data describing a Mg^{2+} binding topography to hammerhead RNA molecules and should be useful for the analysis of other RNA molecules.

Binding of bivalent cations, in particular Mg^{2+}, is essential for the catalytic activity of RNAs (Cech, 1990; Symons, 1992; Bratty et al., 1993; Pyle, 1993). The interaction of these ions with hammerhead ribozymes in solution has been characterized by measurements of the cleavage rate as a function of the divalent metal ion concentration (Perreault et al., 1991; Dahm & Uhlenbeck, 1991; Grasby et al., 1993; Dahm et al., 1993). Metal ion coordination to the cleavage site has been determined by the use of ribozyme substrates containing phosphorothioate substitution at this site (Slim & Gait, 1991; Koizumi & Ohtsuka, 1991; Dahm & Uhlenbeck, 1991). Phosphorothioate interference studies have identified several metal ion binding sites in the ribozyme (Ruffner & Uhlenbeck, 1990; Dahm & Uhlenbeck, 1991). The recording of CD spectra of the hammerhead ribozyme as a function of Mg^{2+} yielded a binding constant of approximately 10^4 M^{-1} for this ion (Koizumi & Ohtsuka, 1991). RNA molecules, like the hammerhead ribozyme, can bind a large number of bivalent metal ions, most of which serve to stabilize RNA secondary and tertiary structural elements, but some metal ions, probably only one or two, are involved directly in catalysis (Dahm & Uhlenbeck, 1991; Steitz & Steitz, 1993; Sawata et al., 1995; Kuimelis & McLaughlin, 1995). Techniques such as equilibrium dialysis, gel retardation, or CD spectroscopy can provide information regarding overall binding affinities and global changes of structure upon metal ion binding. However, this is not suitable for detailed analysis of local binding constants, which requires RNA molecules containing a reporter group, at selected positions, that is sensitive to structural changes upon metal ion binding.

A suitable reporter group is the 2-aminopurine ribonucleoside which can readily be incorporated into various sites of the hammerhead ribozyme (Tuschl et al., 1993; Ng et al., 1994). It has a fluorescent chromophore, and the quantum yield of the emission is very sensitive to changes in interactions with the surrounding bases. Most notable is the decrease in fluorescence of 2-aminopurine when it is involved in base stacking interactions in oligo- and polynucleotides (Ward et al., 1969; Scheit & Rackwitz, 1982; Guest et al., 1991). The change in fluorescence of this nucleobase can therefore be used as a sensitive indicator for structural changes in oligonucleotides. In this report, we follow changes in fluorescence upon complex formation between the ribozyme and the substrate. We also determine binding constants for Mg^{2+} and Ca^{2+} based on fluorescence changes in ribozyme−substrate constructs for cis-cleavage in an effort to characterize the metal ion binding topology.

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MATERIALS AND METHODS

The hammerhead RNA molecules were synthesized by the solid phase method and purified as previously described (Tuschl et al., 1993). The deprotected oligoribonucleotides were desalted on Sep-Pak reverse phase cartridges and taken up in doubly distilled water. Kinetic parameters for ribozymes and substrates modified with 2-aminopurine were obtained from trans-cleaving ribozyme−substrate sequences from Eadie−Hofstee plots using initial velocities under multiple turnover conditions with 5′-^{32}P-labeled substrate. Product and substrate were separated by denaturing 20% polyacrylamide (8 M urea) gel electrophoresis (Tuschl et al., 1993).

The fluorescence of 2-aminopurine-substituted substrate and ribozyme was measured as described below in 50 mM cacodylate, pH 7.5, containing either 50 mM NaCl, a mixture of 50 mM NaCl and 10 mM $MgCl_2$, or 10 mM $MgCl_2$ alone. Concentrations for measurements with 2-aminopurine-substituted substrate were 500 nM dC,2AP-substrate, 2.5 μM ribozyme, or 2.5 μM complementary oligoribonucleotide; for measurements with 2-aminopurine-substituted ribozyme: 500 nM 2AP-ribozyme and 2.5 μM dC-substrate.

The ribozyme−substrate constructs for cis-cleavage used for the determination of binding constants were prepared for the fluorescence measurements in either buffer T (75 mM Tris-HCl, pH 7.5, ionic strength $I = 63$ mM) or buffer TCN (0.1 M $NaCl0_4$, 50 mM Tris−cacodylic acid, pH 7.2, $I =$ 145 mM). Measurements with the latter buffer were performed as a basis for T-jump experiments. The fluorescence intensity was measured using an SLM 8000 S spectrofluorometer connected to a PC which was used for data collection and averaging. The fluorescence was excited at 320 nm with a bandwidth of 1 nm, and the emission was measured at 380 nm with a bandwidth of 16 nm. All measurements were performed at 20.0 °C. Before the measurements, all solutions were centrifuged within the cuvette in a low-speed desk-top centrifuge for 2 min, in order to clear solutions from residual dust particles. Then the cuvettes were thermostated in the cuvette holder of the SLM for 5 min, before fluorescence intensities were measured for a period of 400 s. The fluorescence intensities measured by this procedure never revealed any indication of a slow process. After correction by reference measurements (cf. Results), the data were fitted by a least-squares procedure using the facilities of the Gesellschaft für wissenschaftliche Datenverarbeitung mbH, Göttingen.

Equations used in the fitting procedure: (1) the one-step binding process was described according to the simple law of mass action; (2) the two-step binding process was evaluated by numerical solution of the equation $K_1K_2L^3 + (K_2 + K_1K_2B_o - K_1K_2L_o + K_1 + K_1K_2A_o)L^2 + (K_2B_o - K_2L_o + 1 - K_1L_o + K_1A_o)\,L - L_o = 0$, where L is the free ligand concentration, A_o and B_o are the total concentrations of the two sites, and K_1 and K_2 are the two binding constants. Note that under the conditions of the present experiments, $L_o \gg A_o$ and $L_o \gg B_o$; thus, the values of A_o and B_o do not matter as long as both are much smaller than L_o. The free ligand concentration L was used to calculate the concentrations of all other species, and finally the contribution of all species to the fluorescence intensity was calculated using the relative quantum yields q_1, q_2, and q_{12} for complexes 1 and 2 and the complex with both sites occupied, respectively. We assume that quenching in complexes 1 and 2 can be described by simple elementary rate constants of quenching (cf. textbooks of fluorescence spectroscopy) and that these processes act together in the mixed complex. Under these conditions, q_{12} can be calculated from q_1 and q_2 according to $q_{12} = q_1q_2/(q_1 + q_2 - q_1q_2)$, and thus the number of fitted quenching parameters is reduced from 3 to 2.

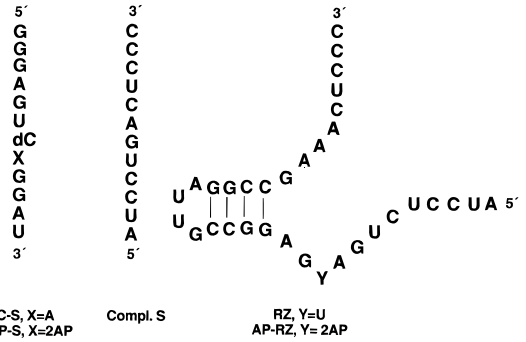

FIGURE 1: Oligoribonucleotide constructs used for monitoring complex formation between ribozyme and substrate. The letters A, U, G, and C denote the standard ribonucleosides; dC ≡ 2′-deoxyribocytidine; 2AP ≡ 2-aminopurine ribonucleoside.

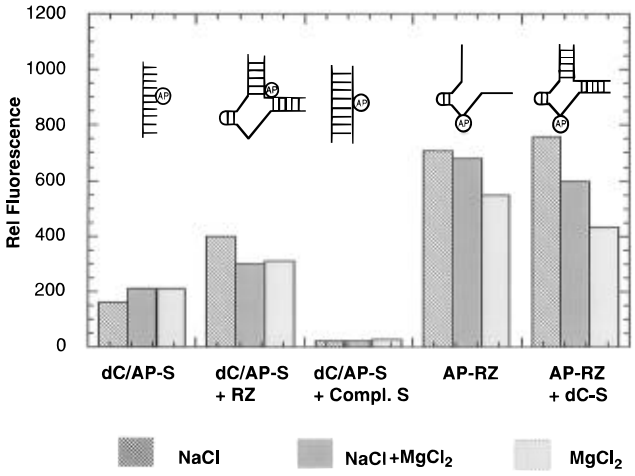

FIGURE 2: Fluorescence changes upon complex formation between ribozyme and substrate. Conditions as described under Materials and Methods. Abbreviations as in Figure 1.

RESULTS

In order to determine the usefulness of the 2-aminopurine nucleoside to monitor conformational changes in the ribozyme−substrate complex, 2-aminopurine riboside was incorporated in the substrate as well as in the ribozyme (Figure 1). Fluorescence changes upon complex formation between substituted substrate and unmodified ribozyme as well as between the substituted ribozyme and unmodified substrate were monitored under varying salt conditions. The 2-aminopurine-substituted substrate (dC/AP-S), with a deoxycytidine at the potential cleavage site, showed essentially the same fluorescence in either NaCl, a mixture of NaCl and $MgCl_2$, or $MgCl_2$ alone (Figure 2). This fluorescence was drastically quenched when annealed to the complementary oligoribonucleotide. This is expected when the 2-aminopurine is stacked in a perfect helix. When the 2-aminopurine-substituted substrate was complexed with the ribozyme (RZ), the fluorescence was increased 2.5 times over the substrate alone in NaCl and 1.5 times when Mg^{2+} was present. The ribozyme with a 2-aminopurine at position 7 (AP-RZ)

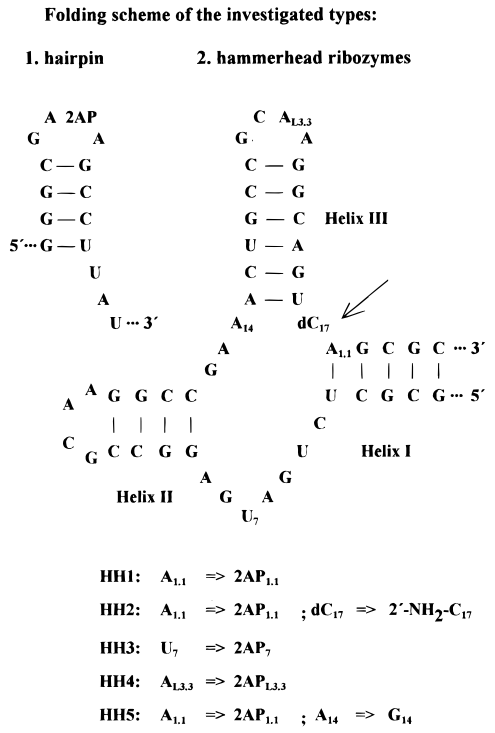

FIGURE 3: Folding scheme of the hairpin loop HP (upper left side) and of the hammerhead ribozymes. The hammerhead ribozymes are substituted by 2-aminopurine at the following sites: the core position 17 is substituted by 2′-deoxycytidine in HH1, HH3, HH4, and HH5, whereas 2′-deoxy-2′-aminocytidine is substituted in HH2; the core position 1.1 is substituted by 2-aminopurine in HH1, HH2, and HH5; the core position 7 is substituted by 2-aminopurine in HH3; the loop position L3.3 is substituted by 2-aminopurine in HH4; the core position 14 is substituted by guanosine in HH5 [numbering according to Hertel et al. (1992)]. Letters as explained in the legend to Figure 1; 2′-NH_2C, 2′-deoxy-2′-aminocytidine.

exhibited a quite high fluorescence which was reduced upon complex formation with the substrate containing deoxycytidine at the cleavage site (dC-S) in the presence of Mg^{2+}.

The hammerhead ribozymes used for the determination of binding constants were cis-cleaving constructs in which cleavage was inhibited by the presence either of 2′-deoxycytidine at position 17 for hammerheads HH1, HH3, HH4, and HH5 or of 2′-deoxy-2′-aminocytidine for HH2 (Figure 3). 2-Aminopurine residues were substituted at position 1.1 next to the cleavage site in HH1, HH2, and HH5; at the single variable core position 7 in HH3; and at position L3.3 in loop III in HH4. Ribozyme HH5 was additionally modified at core position 14, where an adenosine was exchanged by a guanosine, totally abolishing cleavage activity (Rueffner & Uhlenbeck, 1990).

Precise cleavage parameters for self-cleaving constructs could not be obtained by following the appearance of radioactively labeled product since the introduction of the terminal ^{32}P label using either T4 polynucleotide kinase or RNA ligase led to considerable cleavage. When such phosphorylation of an unmodified self-cleaving construct was carried out with T4 polynucleotide kinase in the presence of 10 mM $MgCl_2$, at an initial ribozyme concentration of 1 mM, the phosphorylation was complete after 2 min. However, full-length product accounted only for 30% of total radioactivity; the rest was attributed to cleavage product. It can therefore be assumed that the self-cleaving sequences

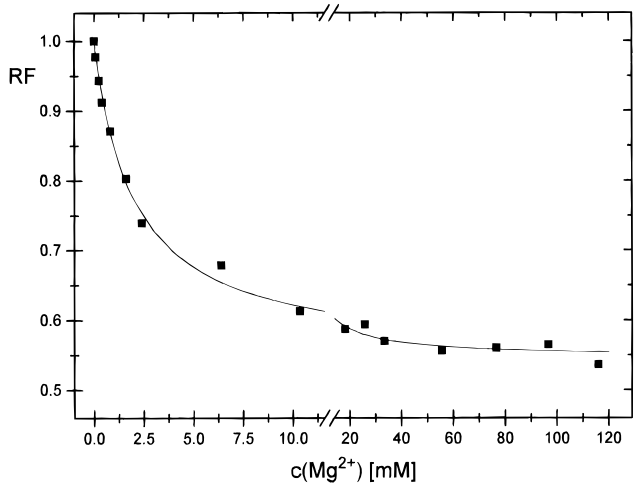

FIGURE 4: Relative fluorescence intensity (RF) of the hammerhead ribozyme HH2 as a function of the Mg^{2+} concentration, $c(Mg^{2+})$. The continuous line represents a least-squares fit by the model assuming a single binding site: binding constant $K = 505\ M^{-1}$, quantum yield of the complex relative to that of the free ribozyme = 0.536 (0.1 M $NaClO_4$, 50 mM Tris/cacodylate, pH 7.2, 20 °C, ribozyme concentration 0.3 mM).

used in this study are folded in the hammerhead conformation.

Michaelis−Menten parameters for the unmodified trans-cleaving ribozyme, acting on an unmodifed substrate, were $k_{cat} = 3.9\ min^{-1}$, K_m 93 nM, and $k_{cat}/K_m = 42.5\ \mu M^{-1}\ min^{-1}$. For the substrate containing 2-aminopurine ribonucleoside at position 1.1, the parameters were k_{cat} 3.4 min^{-1}, $K_m = 71$ nM, and $k_{cat}/K_m = 48\ \mu M^{-1}\ min^{-1}$. 2-Aminopurine substitution at position 17 rendered the substrate uncleavable. Incorporation of 2-aminopurine riboside at position 7 of the ribozyme strand gave an observed rate constant, $k_{obs} = 1.0\ min^{-1}$, at 500 nM substrate saturating concentration (i.e., $k_{obs} = k_{cat}$), which was only a factor of 4 lower than the k_{cat} value for the unmodified ribozyme.

The fluorescence of the various hammerhead constructs was measured in Tris buffer, pH 7.5, which is commonly used for activity measurements, thus facilitating the direct comparison of results. The intensity of the emitted light at hammerhead concentrations between 0.25 and 0.3 mM was rather low. Under these conditions, changes of the background intensity resulting from the addition of Mg^{2+} or Ca^{2+} ions to the buffer, up to concentrations of 0.1 M, were of the same order of magnitude as the change of the hammerhead fluorescence. Thus, in all cases, the data obtained for the hammerhead emission had to be corrected by data obtained for the buffer in a parallel titration experiment. Furthermore, the data had to be corrected for the dilution resulting from the addition of Mg^{2+} or Ca^{2+} ions. A sample set of data obtained after corrections is shown in Figure 4.

Hammerhead ribozyme stock solutions used in this investigation were checked for contamination with bivalent metal ions by monitoring changes in fluorescence intensity on addition of EDTA. No changes were observed within the limits of experimental accuracy, and it was concluded that the concentration of Mg^{2+} or Ca^{2+} ions was negligible.

The most simple interpretation of the experimental data is to assume that there are individual binding sites, which are occupied according to the reaction scheme:

$$\text{site} + \text{ion} \leftrightarrow \text{complex} \qquad (1)$$

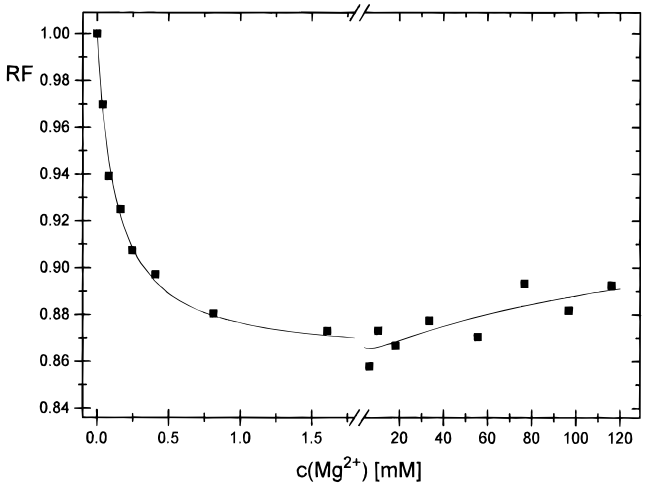

FIGURE 5: Relative fluorescence intensity (RF) of the hammerhead ribozyme HH4 as a function of the Mg^{2+} concentration, $c(Mg^{2+})$. The continuous line represents a least-squares fit by the model assuming two binding sites: $K_1 = 7.7 \times 10^3$ M^{-1}, $K_2 = 7$ M^{-1}, quantum yields of complexes 1 and 2 relative to that of the free ribozyme = 0.86 and 1.071, respectively (75 mM Tris/HCl, pH 7.5, 20 °C, ribozyme concentration 0.3 mM).

with a binding constant

$$K = [\text{complex}]/[\text{site}]\,[\text{ion}] \qquad (2)$$

where [complex], [site], and [ion] denote the equilibrium concentrations of the respective molecular species. The fluorescence intensity was assumed to be a linear function of the degree of binding. Based on these assumptions, the experimental data have been evaluated by a least-squares minimization routine. In most cases, the data could be fitted by the simple model at a satisfactory degree of accuracy (Figure 4).

In a few cases, the accuracy of the data representation by the simple model was not sufficient (Figure 5). In such cases, the analysis was extended using a more complex model with two independent binding sites:

$$\text{site 1} + \text{ion} \leftrightarrow \text{complex 1} \qquad (3a)$$

$$\text{site 2} + \text{ion} \leftrightarrow \text{complex 2} \qquad (3b)$$

We have assumed that the two binding sites are occupied simultaneously and independently according to the simple law of mass action. The change of the fluorescence intensity for each site was also assumed to be independent, with individual degrees of quenching for the two sites, and to be additive upon occupation of both binding sites (cf. Materials and Methods). The equations for the two coupled binding equilibria were solved numerically without approximations using the NAG routine c02agf for evaluation of polynomials. Using this model, it was possible to fit all the data, which could not be represented by the simple model, at a satisfactory accuracy (Figure 5).

The titrations have been performed for Mg^{2+} and for Ca^{2+} ions. The parameters resulting from these measurements are compiled in Table 1. The binding constants for Mg^{2+} range from 1420 to 7580 M^{-1} in T buffer and from 510 to 1610 M^{-1} in TCN buffer. A second binding constant for Mg^{2+} was found for ribozymes HH4 and HH5 with values between 12 and 72 M^{-1}. The difference in binding constants in the two buffers is mainly due to the change in the ionic strength from 63 mM for buffer T to 145 mM for buffer TCN. The binding constants for Ca^{2+} are from 440 to 1010 M^{-1} for buffer TCN. The decreases in fluorescence are in the range up to 52%. For HH4, the change in fluorescence in TCN buffer is close to 0. However, this lack of a change in fluorescence does not necessarily indicate a lack of Mg^{2+} binding in this region.

As a reference, we have also analyzed a simple hairpin loop (cf. Figure 3) with a structure related to that of loop and helix III of the hammerhead ribozyme HH4 (Heus & Pardi, 1991). The titration with Mg^{2+} revealed a relatively small reduction of the fluorescence intensity, which required two binding processes with the parameters $K_1 = 500$ M^{-1}, $K_2 = 78$ M^{-1}, $q_1 = 0.75$, and $q_2 = 1.29$ for a satisfactory fit. Thus, the binding parameters are of the same order of magnitude as those obtained for the hammerhead ribozymes.

DISCUSSION

Metal ion binding on hammerhead RNAs has been studied by a variety of different methods. Besides the kinetic studies for the metal ion dependence of cleavage (Perreault et al., 1991; Dahm & Uhlenbeck, 1991; Grasby et al., 1993; Dahm et al., 1993), the studies with substrates containing chiral phosphorothioate substitution at the cleavage site have provided the most detailed picture for metal ion binding at the cleavage site (Slim & Gait, 1991; Koizumi & Ohtsuka, 1991; Dahm & Uhlenbeck, 1991). The results of these investigations indicate that a Mg^{2+} ion is bound to the *pro-*R_p oxygen of the phosphate to be cleaved. Koizumi and Ohtsuka (1991) postulate that this binding represents outer-sphere complexation on the basis of a binding constant of 10^4 M^{-1}. This metal ion binding site next to the cleavage site has also been observed in the X-ray study by Scott et al. (1995). Additional metal ion binding sites could be identified by phosphorothioate interference experiments. Phosphorothioates of the R_p configuration were randomly incorporated by transcription into a self-cleaving ribozyme (Ruffner & Uhlenbeck, 1990). Separation of cleaved from uncleaved ribozymes allowed the identification of phosphorothioates at the GA double mismatch as interfering with cleavage in the presence of Mg^{2+}. Activity could be restored with Mn^{2+}, indicating metal ion binding to the *pro-R* oxygen at these positions (Uhlenbeck, 1995). One of these sites has also been identified by the X-ray structural determinations (Pley et al., 1994; Scott et al., 1995) along with three other potential metal ion binding sites. These X-ray assignments have to be taken with care since the resolution is not high enough to make these identifications unambiguous. Bassi et al. (1995) propose, on the basis of uranyl photocleavage, a metal ion binding site at position G5 in the central core. They also showed that the presence of Mg^{2+} influences the gel electrophoretic mobility of the ribozyme and therefore global folding. Mg^{2+}-dependent global structural changes could also be followed by changes in fluorescence resonance energy transfer efficiencies (Tuschl et al., 1994; Tuschl and Eckstein, unpublished results). ^{1}H NMR spectroscopy also revealed a Mg^{2+}-dependent folding of the ribozyme−substrate complex (Orita et al., 1995).

The approach adopted here for the determination of affinity constants of Mg^{2+} and Ca^{2+} ions differs from previous methods in that it is based on changes in fluorescence of a 2-aminopurine nucleoside incorporated at certain positions

Table 1: Fluorescence Titrations of Hammerhead Ribozymes (HH1−5) and Hairpin (HP) with $MgCl_2$ and $CaCl_2$[a]

			fit 1		fit 2			
RNA type	metal ion	buffer	K_{Mg} (L/mol)	q_1	$K_{1_{Mg}}$ (L/mol)	$K_{2_{Mg}}$ (L/mol)	q_1	q_2
HH1	Mg	TCN	1610 ± 250	0.74 ± 0.02	–	–	–	–
	Ca	TCN	1010 ± 190	0.91 ± 0.03	–	–	–	–
2AP1.1								
dC17	Mg	T	2930 ± 170	0.66 ± 0.07	–	–	–	–
HH2	Mg	TCN	510 ± 10	0.60 ± 0.05	–	–	–	–
	Ca	TCN	680 ± 140	0.66 ± 0.02	–	–	–	–
2AP1.1					–	–	–	–
2′-NH_2C17	Mg	T	1420 ± 130	0.48 ± 0.01	–	–	–	–
HH3	Mg	TCN	970 ± 10	0.59 ± 0.01	–	–	–	–
	Ca	TCN	900 ± 50	0.59 ± 0.01	–	–	–	–
2AP7								
dC17	Mg	T	2640 ± 30	0.51 ± 0.02	–	–	–	–
HH4	Mg	TCN	–	1.00 ± 0.03	–	–	–	–
	Ca	TCN	440 ± 110	0.90 ± 0.01	–	–	–	–
2APL3.3								
dC17	Mg	T	–	–	7580 ± 110	12 ± 6	0.85 ± 0.01	1.07 ± 0.03
HH5	Mg	TCN	–	–	1270 ± 90	22 ± 6	0.72 ± 0.03	0.43 ± 0.06
2AP1.1								
G14	Mg	T	–	–	3060 ± 220	72 ± 2	0.74 ± 0.03	0.49 ± 0.01
dC17								
HP								
	Mg	TCN	–	–	500 ± 270	78 ± 15	0.75 ± 0.05	1.29 ± 0.15
2APL1.3								

[a] The relative quantum yield's (q_1 and q_2 values) represent the change of the fluorescence in the complex relative to that of the free RNA (20 °C; usually the Mg^{2+} and Ca^{2+} concentrations were increased during the titrations up to 120 mM; in the case of HH5 and HP, the concentrations were increased up to 220 mM).

of the hammerhead ribozyme upon metal ion binding. Base stacking is considered the most prominent factor influencing 2-aminopurine fluorescence (Ward et al., 1969; Scheit & Rackwitz, 1982; Guest et al., 1991). Thus, conformational changes of the nucleoside analogue, that induce changes in base stacking, will be associated with a change in fluorescence.

In the first part of this investigation, complex formation between ribozyme and substrate was monitored by changes in 2-aminopurine fluorescence. Such changes were observed with 2-aminopurine-substituted substrate upon complex formation with the ribozyme and, *vice versa*, upon complex formation with 2-aminopurine-substituted ribozyme and substrate (Figure 2). An increase in fluorescence was seen when the 2-aminopurine-substituted substrate was annealed to the ribozyme, indicating formation of a structure less stacked around the 2-aminopurine than in the substrate alone. However, when the 2-aminopurine-substituted ribozyme was annealed to the substrate, a decrease in fluorescence could be observed in the presence of Mg^{2+}. The same relative fluorescence is observed with a substrate with a ribocytidine at the cleavage site (data not shown). This is evidence for a tightening of the structure around the 2-aminopurine in the complex in the presence of this metal ion. These results also demonstrate that the 2-aminopurine riboside is a suitable probe for monitoring conformational changes in the ribozyme. It was therefore used for the second part of this investigation.

Mg^{2+} is supposed to play a dual role in the ribozyme-catalyzed reaction, a structural and a catalytic one. The latter requires Mg^{2+} concentrations in the millimolar range. It is therefore of interest to know what Mg^{2+} concentrations are required for the conformational changes reported by the 2-aminopurine fluorescence changes. For this study, hammerhead ribozyme cis-cleaving constructs with 2-aminopurine incorporated at various sites were examined. As such, this method not only provides affinity constants for the metal ions but also identifies local conformational changes associated with this binding on addition of metal ions. It should be pointed out, however, that these fluorescence changes of the 2-aminopurine do not necessarily identify this nucleotide as the binding site for the metal ion. The observed conformational change of the 2-aminopurine could be the result of transmission of structural or conformational effects from metal ion binding at neighboring positions. It is expected, because of electrostatic coupling, that the binding constants for the metal ions increase with the local concentration of the phosphates in the environment of the binding site. The hammerhead RNA molecules used in this investigation contain 48 phosphate residues, and, thus, the charge density of these molecules is very high. Under these conditions, it is expected that binding of the first ions to hammerhead molecules is supported by this high charge density, resulting in particularly high binding constants, and compensation of the charges upon further binding should lead to a decrease in the binding constants. The electrostatic coupling of ion binding is described by polyelectrolyte theory (Manning, 1978; Record et al., 1978). However, polyelectrolyte theory has been mainly developed for the case of linear polymers, e.g., linear double helices, and cannot be applied in its standard form to cases with a complex tertiary folding like hammerhead RNAs. For this reason, we have evaluated the experimental data by simple site binding models. Obviously, the resulting binding constants are influenced by polyelectrolyte effects. When an ion binds to a given site, the binding energy is made up by contributions resulting from local coordination contacts and by contributions resulting from electrostatic interactions, which include interactions with charged residues in the more distant environment. These contributions are not separated in our present investigation. Nevertheless, the binding constants should reflect the levels of binding affinities correctly.

The results obtained with the ribozyme HH1 demonstrate a Mg^{2+} binding site, which is indicated by the 2-aminopurine at the cleavage site. These results are consistent with previous data obtained with phosphorothioate modification (Slim & Gait, 1991; Koizumi & Ohtsuka, 1991; Dahm & Uhlenbeck, 1991) and one of the X-ray structures (Scott et al., 1995). The Mg^{2+} affinity is approximately 2.9×10^3 M^{-1}, which is of the same order of magnitude as the 10^4 M^{-1} determined by Koizumi and Ohtsuka (1991). However, the postulation of an outer-sphere coordination will have to be checked by different methods. In addition to the phosphates, metal ion coordination sites may also be provided by 2′-OH groups and the heteroatoms of the bases. In HH2, the presence of the 2′-NH_2 group of cytosine 17, next to the cleavage site, results in a 2-fold reduction in the Mg^{2+} binding affinity compared to that of HH1, from 2.9×10^3 M^{-1} to 1.4×10^3 M^{-1}. The presence of 2′-deoxy-2′-aminocytidine at this position does not support ribozyme cleavage (Pieken et al., 1991). The lack of nucleophilic attack of the 2′-amino group at the phosphorus, which is required for product formation, is under study (Thomson, Patel, and Eckstein, unpublished results). Nevertheless, it was expected that the 2′-amino group might be a better mimic for Mg^{2+} binding than the 2′-deoxy analogue. However, the binding constant for the 2′-amino derivative was only half that of the 2′-deoxy derivative. This result might indicate that the 2′-NH_2 group induces unfavorable structural changes at the cleavage site.

The ribozyme HH5 differs from HH1 in the replacement of adenosine 14 by guanosine. This replacement inactivates the otherwise unmodified hammerhead ribozyme (Ruffner et al., 1990). It was therefore of interest to see whether this replacement would be of consequence for Mg^{2+} binding. The fluorescence change as a result of Mg^{2+} binding could not be fitted to a single binding process. The higher binding constant of the two in T buffer, 3.1×10^3 M^{-1}, was comparable to that found for HH1. Thus, there seems to be no change in Mg^{2+} binding conferred by this replacement. Bassi et al. (1995) had found by gel electrophoretic mobility analysis for the same mutant that there was no Mg^{2+}-dependent global folding. Thus, it is clear that we are monitoring a conformational change which does not manifest itself in the global folding of this inactivated ribozyme.

Somewhat surprising is the result obtained with HH3 where uridine 7 is replaced by 2-aminopurine riboside. This position is not invariant and can actually be replaced by an abasic nucleotide (Beigelman et al., 1995). We find a Mg^{2+} binding site indicated by the 2-aminopurine at this position with an affinity similar to that indicated by the 2-aminopurine at the cleavage site, which is associated with the strongest fluorescence change seen in this study.

For HH4, with a 2-aminopurine located in loop III, the Mg^{2+} binding in T buffer could again not be simulated by a single binding process, thus indicating two binding constants. The first of these is the highest found in this study, which is surprising as the phosphate density is not particularly high in loop regions. The binding constants not only are influenced by polyelectrolyte effects but also may be affected by changes of the structure upon ion binding. If ion binding is coupled with the formation of some secondary or tertiary structure, the observed binding constant is increased by a factor $(1 + K_t)$, where K_t is the equilibrium constant for the formation of this structure. The binding of Mg^{2+} to loop III is not considered important for the function of the ribozyme as the presence of this loop is not a prerequisite for activity.

For comparison, we have also analyzed the hairpin loop HP which is in the same GNRA-tetraloop family as loop III of HH4. The difference in the fluorescence change observed for HH4 and HP indicates a different Mg^{2+}-induced change of the conformation although the overall structure characteristics of a G/A mismatch in the loop should not have been affected by the loop sequence variation (Heus & Pardi, 1991). The lower first Mg^{2+} binding constant of the hairpin compared to the ribozyme HH4 may also reflect a reduced contribution from electrostatic attraction in the isolated hairpin. Apparently small changes in the environment of the fluorescent probe may lead to rather large changes of Mg^{2+} binding constants and of Mg^{2+}-induced changes of conformation.

The set of data obtained by these measurements demonstrates the existence of a broad distribution of Mg^{2+} binding processes for the hammerhead ribozyme, as should be expected for any large RNA molecule. Thus, a broad distribution of Mg^{2+} binding processes has been shown previously for tRNAs (Teeter et al., 1980), and some of these binding sites have been identified by X-ray crystallography (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980). The binding constants for strong binding sites for metal ions in tRNAs are of the order of 10^4 M^{-1} (Cohn et al., 1969; Stein & Crothers, 1976; Bina-Stein & Stein, 1976; Saenger, 1983). The binding constants of Mg^{2+} and Ca^{2+} ions for the hammerhead ribozyme indicated by 2-aminopurine fluorescence for the first binding step are on the order of 10^3 M^{-1} and are thus lower than for tRNA but higher than those evaluated from the catalytic reaction. The constants obtained for the second binding step—wherever a second step has been detected by the 2-aminopurine fluorescence—are lower than those found for the sites affecting catalysis. Perreault et al. (1991) derived apparent binding constants for Mg^{2+} ions involved in the catalysis from measurements of the cleavage rate a different Mg^{2+} concentrations. The binding constants found in their investigation for various hammerhead constructs in buffer T are in the range around 100 M^{-1}. Thus, the ions involved in catalysis are of intermediate affinity. As has been pointed out, Mg^{2+} ions play a dual role in stabilizing the tertiary structure and in participating directly in the cleavage mechanism (Dahm & Uhlenbeck, 1991; Long et al., 1995). The strong binding of Mg^{2+} and Ca^{2+} ions shown in this study presumably identifies the affinity of metal ions for maintaining the tertiary structure.

In the X-ray structures of the hammerhead ribozyme the 2′-OH group at the cleavage site is not oriented in such a way that it can attack the phosphorus in an in-line mechanism to produce the cyclic phosphate with inversion of configuration (Pley et al., 1994; Scott et al., 1995). A conformational change is required to obtain the proper alignment of these two atoms for the cleavage reaction to proceed. It is thus of interest to find that the binding of Mg^{2+} and Ca^{2+} ions close to the active site is associated with a conformational change.

This study establishes binding constants of Mg^{2+} and Ca^{2+} ions for the hammerhead ribozyme other than by the kinetics of cleavage and identifies conformational changes associated with this binding. The results provide information that is

complementary to that obtained previously from X-ray crystallography and from phosphorothioate interference experiments. More information on the ion coordination to catalytic RNAs may be expected from investigations of the kinetics of metal ion binding, in particular to identify inner-sphere complexes (Porschke, 1995). The procedure described in the present investigation should be useful for this purpose, and such investigations are in progress.

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