NMR study on the impact of metal ion binding and deoxynucleotide substitution upon local structure and stability of a small ribozyme

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Abstract We have studied a very small ribozyme described earlier which requires the presence of soft metal ions like manganese or cadmium. It consists of only three uridines as ribozyme, cleaving the sequence 5′-GAAA-3′ after the guanosine. We have set out to characterize the metal ion binding in this system by NMR spectroscopy and the impact of the ribose 2′-OH group of the cleavable nucleotide upon local structure. NMR results indicate a high degree of regularity and order in the pyrimidine-rich ribozyme strand, and high flexibility within the purine-rich substrate. The guanosine 2′-hydroxy group adjacent to the cleavage site was found to have a profound effect upon the structure, apparently destabilizing a stacked arrangement. Metal ions were found to bind in a rather unspecific way, however, in the presence of higher amounts of divalent ions a preference in the vicinity of the cleavage site could be observed. ^{113}Cd NMR spectra suggest a specific binding of Cd^{2+} ions to the RNA.

Key words: Nuclear magnetic resonance; RNA; Ribozyme; Metal ion

1. Introduction

Metal ions play a crucial role in the catalytic activity of all ribozymes known so far. These have thus been regarded as a special class of metalloenzymes [1,2]. Knowing the way of complexation of the metal ion is an important step towards an understanding of the reaction mechanism. A number of approaches to monitor the binding of metal ions to nucleic acids and to derive models for the ribozymatic cleavage have been described, either by X-ray crystallography [3–7], by biochemical techniques such as the 'manganese rescue' experiment [8], or by NMR spectroscopy. Here, the use of paramagnetic ions and the observation of the line broadening caused by them is the most straightforward one [9,10], whereas the observation of metal NMR spectra [11] is less popular for nucleic acids.

The structural and mechanistic characterization of ribozymes by the use of NMR techniques was successful only in a small number of examples. Yet there are detailed studies on the conformation of a lead-dependent ribozyme ('leadzyme' [12]) and of the hairpin ribozyme [13], whereas the hammerhead ribozyme up to now does not permit a complete structural characterization by NMR [14,15]. However, NMR has proven to be suitable for the localization of metal ion binding sites in ribozyme fragments. Use of the line broadening effect of paramagnetic ions [16], or of NOEs to the ligand sphere [17] led to the identification of metal ion binding sites in fragments of the self-splicing intron from *Tetrahymena thermophila*. We have used a much smaller ribozyme derived originally from the *Tetrahymena* intron [18], which had been reduced systematically to yield the smallest ribozyme known so far [19]. This consists only of a $(U)_3$ as ribozyme strand, cleaving the sequence 5′-GAAA-3′ specifically after the guanosine residue, and being dependent on soft metal ions such as manganese or cadmium. However, it is inactive with ions like magnesium which are required by most ribozymes. The smallness of this system resembles homogeneous metal catalysts employed in industrial processes and makes this an ideal object for detailed studies on the basic principles of ribozymatic catalysis mechanisms.

Kazakov and Altman [19] presented a model for the mode of ion complexation, taking into account the binding preference of soft metal ions to the nitrogens of purine bases. In this model, the track of four consecutive purines is wrapped around the ion (actually the involvement of two ions is postulated), thus creating a suitable environment for the ion to induce cleavage of the phosphodiester bond. However, there is no direct structural evidence for the occurrence of this structure. One objective has thus been to prove – or disprove – this model by means of NMR spectroscopy. Moreover, the implications of the 2′-OH group of the ribose of the cleaved-off nucleotide, which has been demonstrated before to be of crucial importance in the cleavage reaction, with respect to local structure and conformation of the ribozyme should be analyzed by comparing the all-RNA duplex and a variant having a deoxy-G at the cleavage site.

2. Materials and methods

The two sequences under investigation (Fig. 1) were synthesized chemically using the H-phosphonate method according to Arnold et al. [20]. After deprotection, the RNA was purified with a DEAE ion exchange column. The full-length ribooligonucleotide was obtained by HPLC on a Nucleogen 500-7-DEAE anion exchange column (Macherey and Nagel), using KCl gradients under denaturing conditions (7 M urea, 60°C), and subsequently desalted on a Biogel P6 column. RNA content was checked in all cases using UV absorption [21]. The enzymatic activity was checked using the published procedure [18] with 5′-^{32}P-labelled RNA (data not shown).

NMR samples of A and B were dissolved in a buffer system of 20 mM sodium phosphate, pH 6.5, and 100 mM sodium chloride. RNA concentrations ranged from 0.2 to 1.0 mM. NMR spectra of exchangeable protons were measured in 9:1 H_2O:D_2O; all other NMR spectra were measured in 99.996% isotopically pure D_2O. DSS (sodium-2,2-dimethyl-2-silapentane-5-sulfonate) was used as internal reference. Other metal ions were added as concentrated stock solutions. In all cases when cadmium ions were employed, the phosphate buffer had to be replaced by a citrate buffer to avoid precipitation of insoluble cadmium phosphates.

NMR spectra were recorded on a Bruker DRX-500 spectrometer at a proton frequency of 500.13 MHz under control of a Bruker Aspect-Station. Temperature was adjusted with a Bruker thermostat in con-

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junction with a Haake cryostat bath. The solvent signal was suppressed either by presaturation, or by a 1331-binomial sequence [22] for detection of exchangeable proton signals. NOESY spectra were recorded in phase-sensitive mode in F1 using the TPPI technique [23] with 4096×512 complex data points in F2 and F1, respectively.

Data were processed on an SGI Indigo^2 computer using the NDEE program package (Software Symbiose, Bayreuth). One-dimensional spectra were apodized by exponential multiplication (1 Hz line broadening), two-dimensional spectra by use of a $\pi/2$ shifted squared sinebell function. In all cases, a baseline correction was applied.

3. Results

3.1. Effect of metal ions upon imino proton resonances

To obtain appropriately stable RNA duplexes (with sufficiently high melting temperatures), the minimum structure consisting of only three A-U base pairs [19] was extended by appending several nucleotides to either strand. The resulting duplexes are depicted in Fig. 1, the minimum structure being marked in boldface. The sequence variant A is mainly a section of the original intron sequence [18], while sequence variant B represents the minimum structure with two additional stabilizing C-G pairs and a 3′-dangling adenosine on the substrate strand.

The detection of imino proton resonances allows an easy but accurate assessment of the secondary structure [24]. Sequence-specific assignment of the imino signals was performed using one-dimensional NOE difference spectroscopy.

Addition of sub-stoichiometric amounts of manganese (40 μM and 80 μM, respectively/1 mM RNA) to sample solutions of variants A and B resulted in line broadening effects which were fairly weak as compared to the effects observed for specific manganese binding to RNA molecules of similar size [9,10,16]. The paramagnetism of manganese should give rise to a specific line broadening for the resonances of imino protons in the vicinity of these ions, i.e. near the binding sites.

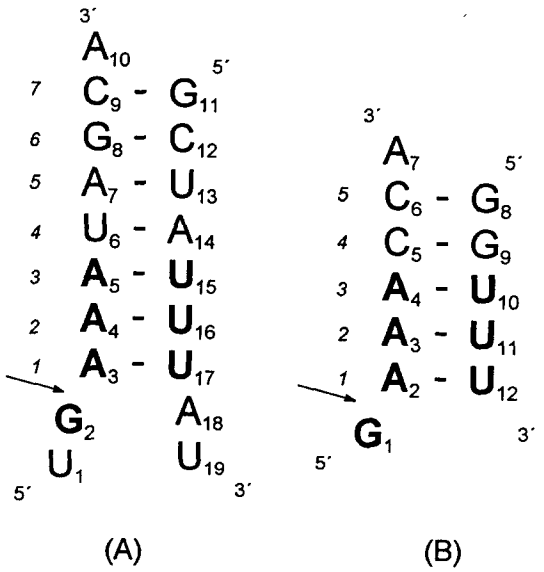

Fig. 1. Secondary structures of the two ribooligonucleotides under investigation. The two sequence variants are marked A and B, respectively. The numbering scheme of the nucleotides is indicated, as well as the counting of Watson-Crick interactions (in italics) referred to in the imino proton spectra. The minimum sequence [19] is displayed in boldface. Both sequences contain additional Watson-Crick base pairs to enhance stability and a 3′-dangling adenosine to strengthen interstrand interactions [37].

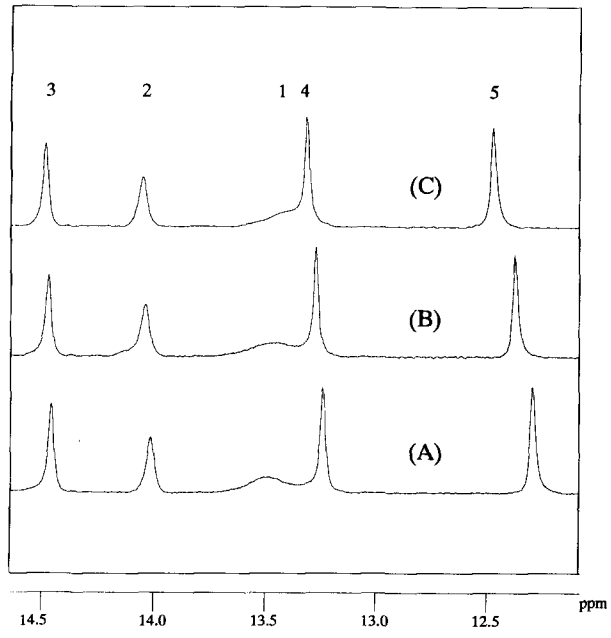

Fig. 2. Effect of cadmium ions on the resonance positions in the imino proton spectrum of sequence variant A (1 mM). Solutions contained 10 mM sodium citrate buffer pH 6.5, 100 mM NaCl and varying amounts of $Cd(NO_3)_2$: (A) no cadmium, (B) 2 mM, (C) 10 mM. Spectra were measured in 90% H_2O/10% D_2O at 268 K.

Instead of a specific effect, as originally expected for a manganese-dependent ribozyme, a general line broadening for all lines (except for the one from the first base pair) was found. However, this effect is easily explainable due to the charge shielding properties of divalent ions, which are known to lead to preferred binding of these ions to the phosphate backbone of nucleic acids. This also explains the decrease in linewidth for the first base pair; here, the high flexibility is decreased by the binding, thereby giving rise to base pair stabilization and slower exchange with the solvent, which in its turn leads to the observed line narrowing.

If an excess of magnesium ions (about 100-fold over manganese) is added, these unspecific binding sites are saturated with magnesium ions, and manganese is expected to migrate preferably to the more manganese-specific binding sites. The strongest – although still fairly weak – line broadening effect is then observed for the first and second of the three A-U base pairs (linewidth increases of ca. 23 and 8 Hz, respectively, for variant B). The results are very similar for both variants (A and B).

In summary, small line broadening effects upon addition of sub-stoichiometric amounts of Mn^{2+} to the RNA are detected, though they are much less pronounced than expected for a specific binding of divalent cations which definitely play a crucial role in the catalytic cleavage of a well-defined phosphodiester bond between two distinct nucleotides.

The observed line broadening could thus be caused by a specifically (though weakly) bound paramagnetic ion, as demonstrated in the studies of Limmer et al. [9], Ott et al. [10], or Allain and Varani [16]. However, the alternative view that the weakening of the base pair by complexation (e.g. by the aforementioned 'wrapping' of RNA around the ion) causes the line broadening is also conceivable. To check this possibility, the effect of cadmium was studied, which likewise mediates catalytic activity [19] without being paramagnetic. It is thus pos-

sible to use a comparatively high concentration without the necessity for additional divalent cations, such as magnesium.

The effect of 2 mM and 10 mM cadmium on the spectrum of sequence variant B is shown in Fig. 2. The uracil (U12) imino resonance of the first base pair shifts markedly upfield upon addition of Cd^{2+} ions. By contrast, the resonances of stem iminos are shifted downfield. Since the large downfield shift, especially for G8 and G9 (base pairs 5 and 4, respectively; cf. Fig. 1) imino resonances, is not observed for the aromatic protons of C5, C6, G8 or G9 (data not shown), it cannot be due to structural changes brought about by the ion. Instead, the exchange rates must be taken in account. A stabilization of a base pair causes a reduced rate of proton exchange with water and a chemical shift change to lower field. The shift change reflects the same stabilizing effect due to the shielding of charges as found with magnesium, albeit much stronger.

3.2. *Effect of cadmium upon the non-exchangeable proton resonances*

In the presence of cadmium ions, some resonances are shifted in the resulting NMR spectra, and these can easily be assigned using two-dimensional NMR spectroscopy. After addition of 8 mM cadmium (RNA concentration 1 mM), only two NOESY cross peaks experience a noticeable shift (data not shown), namely the intraresidual H1′-H8 cross peaks belonging to the nucleotides G1 and A2. This weak shift change (downfield shifts of ca. 0.04 ppm for A2 H8, and ca. 0.05 ppm for G1 H8) can be considered to be due to specific binding of cadmium, since the same amounts of magnesium do not produce a comparable effect. Furthermore, it can be interpreted in terms of a structural rearrangement in this region. The downfield shift of the G1 resonance and the lack of interresidual NOEs between G1 and A2, even in the uncomplexed form, are compatible with a destacking of this residue relative to A2. The assumption of only minor structural variations after addition of Cd^{2+} is corroborated by the fact that neither are NOEs disappearing, nor are new cross peaks emerging, even the NOE intensities are only slightly changed.

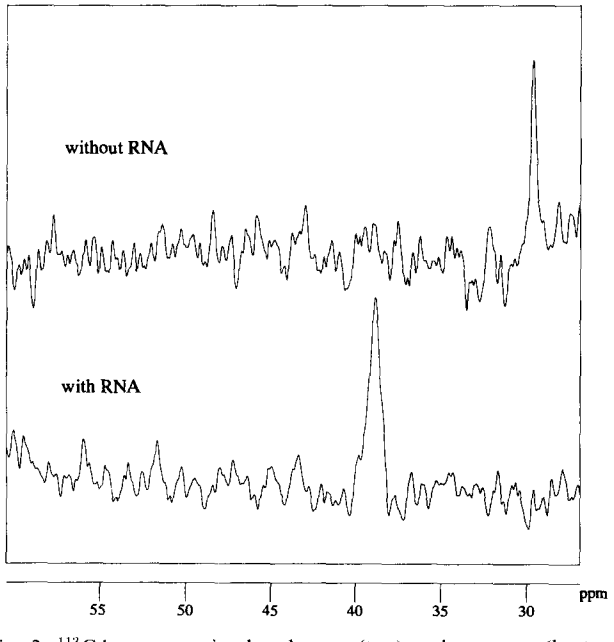

Fig. 3. ^{113}Cd spectrum in the absence (top) and presence (bottom) of the ribozyme, sequence variant B (spectra of 4 mM $Cd(NO_3)_2$ in 10 mM sodium citrate buffer, pH 6.5, 100 mM NaCl, temperature 276 K). Apparently, binding occurs, causing a chemical shift change as well as a distinct broadening of the metal resonance.

Incubation at 40°C for 1 h to promote cleavage of the ribozyme led to the disappearance of the G1 H1′-H8 cross peak, whereas the A2 cross peak was only slightly shifted which is explicable by cleaving-off of the guanosine residue. This finding further corroborates the assumption of a largely destacked arrangement of G1, whose absence apparently has almost no influence on the chemical shift of A2.

3.3. *Cadmium NMR spectra*

Cadmium is a favorable nucleus for NMR spectroscopy, possessing the NMR-active spin 1/2-nucleus ^{113}Cd in 12.26% natural abundance. This nucleus has been used for metal ion binding studies in proteins where it replaced calcium in its specific binding sites [11]. The broad chemical shift range typical of a heavy metal reflects the sensitivity to the complexation status of the ion [25], and is therefore well suited to identify and possibly locate specific binding sites.

In Fig. 3 the 111 MHz ^{113}Cd NMR spectra are shown (10 000 transients, 4 mM $Cd(NO_3)_2$). The lower trace represents the spectrum in 20 mM sodium citrate buffer solution, pH 6.5. Addition of the ribozyme (variant B) has a dramatic effect on the NMR spectrum. The line shifts downfield by ca. 9 ppm and broadens considerably (from 40 to 80 Hz). Since the spectra were run at high molar excess of cadmium over RNA (4 mM versus 0.25 mM), the occurrence of a single line indicates fast exchange ($k_{ex} \geq 10^4\ s^{-1}$) on the NMR timescale. Taking into account the respective concentrations of RNA and metal ions, as well as the small fraction (due to the weak binding) of bound metal ions, the chemical shift change caused by the ion binding should be considerable (in the range of 100 ppm). Such a large variation hints at a specific complexation of the metal by the ribooligonucleotide.

After cleavage of the RNA (1 h at 40°C), the ^{113}Cd NMR spectrum has no discernible peaks (data not shown). The reason is most probably that the signal is too small and too broad to be detectable at the given signal-to-noise ratio. This line broadening might be caused by fast exchange between the different non-specific binding 'sites' for divalent metal ions covering a wide range of chemical shifts, which now takes place. This measurement once more corroborates the before-mentioned statement that the line broadening and the large shift are not due to unspecific binding.

3.4. *Tertiary structure studies*

We have assigned the non-exchangeable protons of the two sequence variants using standard methodology with NOESY and COSY spectra [24]. The inter residual NOEs for sequence variant A are compiled in Fig. 4. A continuous trail of NOEs connecting the anomeric and the aromatic protons can be drawn for the ribozyme strand. Such a pattern is characteristic of helical structures, which in this case showed the typical features of A-RNA. By contrast, the substrate strand lacks these NOESY cross peaks in the region of the cleavage site. There is a clear break in the connectivity between residues U1, G2, A3 and A4, indicating enhanced flexibility and a lack of order in the region of the cleavage site. A weak NOE was found between nucleotides G2 and A4, being indicative of a 'bending back' of the RNA, as was proposed in the original

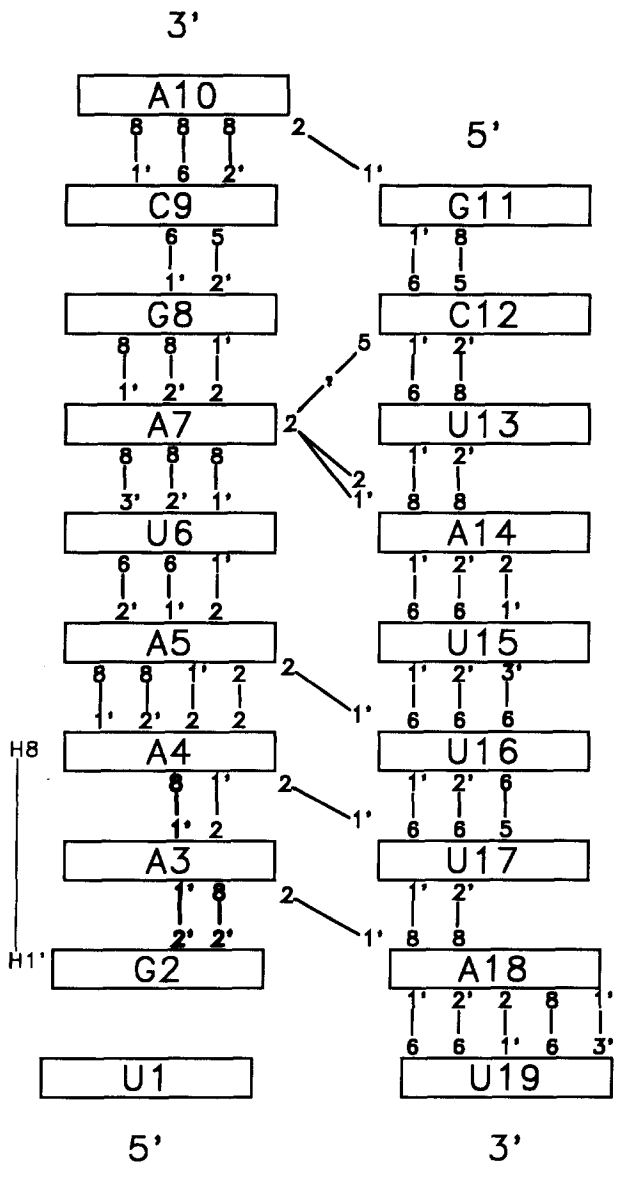

Fig. 4. Interresidual NOE contacts for sequence variant A. Additionally, most of the intraresidual NOEs expected for an A-type RNA conformation were found. All ribose moieties (except the 3'- and 5'-terminal ones) were found to be in the 3'-*endo* conformation. In the 'ribozyme' strand (G11-U19), the NOE connectivities indicate a regular A-type helix, whereas the lack of NOEs in the vicinity of the cleavage site in the 'substrate' strand (U1-A10) suggests enhanced flexibility and/or conformational disorder. A very weak NOE between residues G2 and A4 is consistent with a bending back of the sugar-phosphate backbone in this region, as assumed by Kazakov and Altman [19]. This NOE is not observed with a dG2 variant, whereas additional sequential NOEs (indicated by thicker lines) were detected in this case.

work of Kazakov and Altman [19]. A similar picture was obtained with sequence variant B (data not shown). In this case, however, the weak 'long-range' NOE could not be observed.

The metal ion can be placed within this structure surrounded by purine ring nitrogens. Our results indicate that this structure – which is obviously a rather flexible one – is essentially present already in the absence of metal ions, although there are subtle differences. However, owing to the missing NOEs between the guanine and one of the adenines a well-defined structural model (especially in the vicinity of the cleavage site) cannot be derived. Such a model is not necessarily expected for a molecule of this size, which should display a high degree of intramolecular dynamics (flexibility) and conformational freedom due to the small number of tertiary interactions. A general stabilization of this flexible structure is mediated by the binding of divalent metal ions.

3.5. *Structural effect of a hydroxy group*

A variant of sequence (A) containing a single deoxyguanosine in place of residue G2 showed remarkable differences to the all-RNA ribozyme. The ribose of G2 is assumed to be indispensable for the specific cleavage. Surprisingly, the deoxy-G variant displays a number of sequential interresidual NOEs not present in the all-RNA form, in particular between G2 and A3 (Fig. 4), suggesting a higher degree of stacking order and a better defined (helical) structure in the vicinity of the cleavage site for the deoxy variant. The same picture is obtained by monitoring chemical shift differences between the two variants (Fig. 5). The replacement should give rise to alterations of chemical shifts only for the respective anomeric H1′ proton (beyond, of course, H2′ and H3′ protons, which are not considered in Figs. 4 and 5), if the conformation is not to be affected. All additional changes have thus to be ascribed to alterations of tertiary structure. Actually, large differences are observed in the vicinity of the substituted nucleotide, especially for the track of purines, pointing to a distinctly altered local stacking geometry. This finding is in agreement with the observation of additional sequential NOEs. In particular, the distinct upfield shifts of the aromatic resonances of both A3 and U17 can easily be understood in terms of a more or less regular stacking of dG2 over the base pair A3-U17 (with dG2 overlapping partly with U17).

Apparently, the guanosine 2′-OH group destabilizes a stacked arrangement, and concomitantly favors the less well-ordered structural scheme which is necessary for the complex-

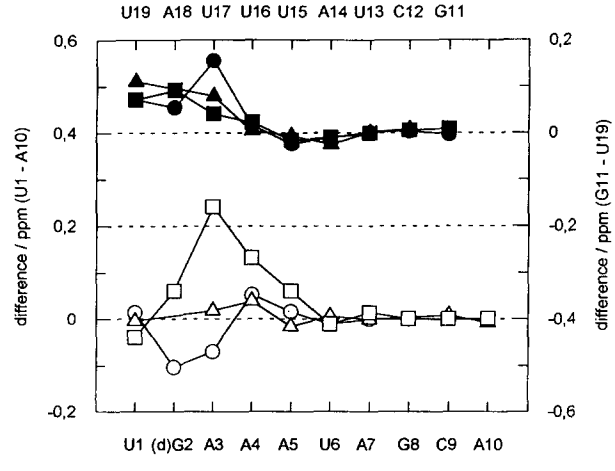

Fig. 5. Comparison of chemical shifts between the all-RNA ribozyme (sequence variant A) and the dG2 variant [$\Delta\delta = \delta$(all-RNA)$-\delta$(deoxy variant)] at 289 K, 10 mM sodium phosphate buffer pH 6.5, 100 mM NaCl, 10 mM $MgCl_2$. Only the H1′ (circles), aromatic H2/H5 (triangle) and the aromatic H6/H8 protons (squares) are shown. The 'ribozyme' sequence (filled symbols) and the 'substrate' sequence (open symbols) display deviations in the vicinity of the substitution site, indicating a structural role for the hydroxy group, since only the H1′ chemical shift of (d)G2 is affected directly by the substitution at the C2′ position.

ation of the metal ion. It is not clear, however, why or how this group destabilizes the stacked arrangement. Possibly, the 2′-OH group is involved in hydrogen bonding of water molecules bridging functional groups of different nucleotides, as seen in crystal structure analyses of RNA duplexes containing G-U wobble pairs (see, e.g. [26], and references cited therein), or in complexing metal ions.

Non-cleavable substrate analogues, such as 2′-deoxy or -methoxy derivatives, are often employed in structural studies of ribozymes (cf. e.g. [4,5]). The comparison of an all-RNA hammerhead ribozyme to an uncleavable analogue [5,6] concerning the metal ion binding behavior showed subtle differences in the cleavage site region. This clearly demonstrates that assertions derived from the analysis of oligonucleotides containing such analogues should be made very carefully and cautiously. These deoxy analogues not only disable the nucleophilic attack of the 2′-OH group to phosphorus but moreover can influence the binding properties of ions, or even the local conformation.

These findings shed new light on the role of the 2′-OH group of the cleaved-off nucleotide. Possibly, the 2′-OH groups are not only essential for the reaction itself, but moreover are important in maintaining and creating a suitable geometry for binding of metal ions and for the catalytic process. Indeed, it was possible to separate such structural implications from the chemical role of the 2′-hydroxy groups in the group II intron active site [27].

4. Discussion

The exchange of metal ions between free and RNA-complexed forms was found to be fast on the NMR timescale, i.e. rate constants are in the order of magnitude of more than 10^3 s^{-1}. The metal ion should, correspondingly, not be bound very tightly, and major conformational changes of the RNA should not be expected, since only a high enthalpy of binding could provide the energy required for a structural rearrangement. This fits into the picture presented above that the binding pocket is preformed already in the absence of the ion. The metal ion is bound in a highly flexible track of purines. This confirms previous findings that the combination of purine-rich regions with structural irregularities is typical of the specific binding sites for soft metal ions [10,16,28].

According to the NMR data, the ribozyme strand displays a high degree of order and is in regular A-helical conformation, whereas the order is far worse for the three adenosine residues in the 'substrate' strand. The guanosine, which is cleaved off in the course of the ribozymatic reaction, is destacked from the adjacent adenosine 3′ of the guanosine, and is involved in the formation of a pocket of three purine residues (G2, A3 and A4). Such a destacked arrangement is also in agreement with the conclusions that can be derived from a comparison between chemical shift data expected for a random coil RNA [29], and the chemical shifts measured for the two RNA duplexes (data not shown). Whereas all residues experience upfield shift changes due to stacking in the well-ordered helix, the G2 chemical shifts resonate at even lower field than expected for the random coil.

Interestingly, in the variant with the non-cleavable deoxyguanosine the guanine base is obviously distinctly better stacked, thus giving rise to a better defined local A-helical geometry and accordingly increased stability in the vicinity of this crucial residue than in the case of the cleavable all-RNA duplex. Possibly, locally enhanced conformational flexibility and/or increased structural disorder could be essential prerequisites for efficient ribozymatic action. The fact that the presence of a G nucleotide at the cleavage site in the hammerhead ribozyme inhibits the cleavage reaction has been explained by the formation of a stable Watson-Crick pair with a conserved C in the core of this ribozyme [15,31–33]. Formation of a stable base pair inevitably restricts the conformational freedom of the nucleotides involved and by this increases the local structural order (conformational rigidity) in the vicinity of the respective residues.

The high degree of order in a track of uridines and, conversely, the high disorder in three consecutive adenosines is, at first sight, contrary to the well-known fact that purines have a higher tendency for stacking. However, the π-systems of the different nucleotides are of very different size, and the spatial requirements for self-stacking of adenosines might be less stringent than for uridines, enabling sufficient stacking for a larger number of possible subconformations. By contrast, for consecutive uridines a constructive stacking interaction might require a better defined arrangement and a higher degree of order.

Very recently, the manganese-dependent ribozyme which was investigated in this work has been characterized biochemically [30]. The binding of platinum complexes, which is known to occur to purine N7, and to be tighter than the binding of manganese, was used to monitor potential binding sites. Only complexation at the A2 or A3 moiety (equivalent to A3 or A4 in sequence variant A) caused suppression of the cleavage activity. Furthermore, all complexes in which platinum is coordinated to more than one nucleotide and thus imposes an unusual, non-A-helical geometry on the RNA were found to be inactive, which confirms our findings that the overall geometry in the $(A)_3{\cdot}(U)_3$ track is not very different from a regular A-helical one.

To elucidate a catalytic mechanism on the basis of a structural model alone is not feasible. A possible reaction pathway can nevertheless be outlined. The base-paired track of three adenines forms, together with the unpaired guanine, a pocket. This binds soft metal ions specifically and raises the local concentration of these ions. Furthermore, the same metal ions are bound at sites distant from the cleavage site. The view of separable 'catalytic' and 'structural' roles for the various ions might therefore be misleading. Indeed, the question arises whether the two can be separated at all for such a small ribooligonucleotide. Recent studies of the hairpin ribozyme [34–36] suggest that the need for metal ions in the catalytic step could be less important than assumed previously, and that the major role of the bound ions is to create a suitable environment, which does not necessarily imply that this structure must be a rigid one. Rather, a whole set or ensemble of energetically similar, but conformationally different substructures could be present. X-ray crystallographic studies of metal ion binding sites for various ions in $tRNA^{Phe}$ [3] and the hammerhead ribozyme [5–7] have revealed a high number of bound ions at various positions, of which only a very small fraction is directly involved in the cleavage reaction.

Another question that has to be addressed is the requirement of the base pairing for the three adenines, which actually occurs. In fact, the minimum ribozyme is rather unusual in that not the ribozyme itself, but rather the substrate provides

the complexation geometry for the catalytically active center, namely the metal ion. The most plausible explanation is that a certain degree of stabilization or conformational rigidity by base pairing is needed. That a stretch of three adenines is necessary may be due to the fact that both the base pairing and the stacking interactions are weaker than for G-C pairs, thus providing an optimal compromise between an adequately well defined molecular geometry on the one hand, and still sufficiently high conformational flexibility (and adaptability) on the other hand.

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