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Proton NMR studies of manganese ion binding to tRNA-derived acceptor arm duplexes

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ABSTRACT

Several RNA duplexes corresponding to the acceptor arms of different tRNAs have been analyzed with respect to their divalent metal ion binding capability by means of proton NMR spectroscopy using paramagnetic Mn^{2+} ions as probes. In particular, the role of GU wobble base pairs has been analyzed with reference to their potential for creating metal ion binding sites. It is shown that both the structural modifications induced by GU pairs in the A-RNA geometry and the sequence context seem to affect the metal ion binding capabilities.

INTRODUCTION

The role of metal ions and especially of Mg^{2+} in the stabilization of the tertiary structure of tRNAs is well-established [1,2]. Beyond this general function of Mg^{2+} ions more specific roles have been discussed for certain magnesium ions bound to well-defined sites in different tRNA regions [3]. In particular, it was suggested that a magnesium ion directly coordinated to phosphate 2 and hydrogen-bonded to phosphate 1 via water molecules at the 5′ end of yeast $tRNA^{Phe}$ acceptor stem could be involved in the binding of the tRNA to both its aminoacyl-tRNA synthetase and the ribosome [3]. A recent study performed in our laboratory [4] on magnesium/manganese ion binding to a 18-nucleotide RNA duplex derived from the *E.coli* $tRNA^{Ala}$ acceptor stem lends further support to this assumption. In this study specific $Mn^{2+}(Mg^{2+})$ binding site located between the second and third basepairs of the stem was detected by means of proton NMR. It seems of special interest in this context that the main identity element of $tRNA^{Ala}$ which is essential for the recognition by its aminoacyl-tRNA synthetase, the G3-U70 base pair [5,6], is in the immediate neighbourhood of this specific metal ion binding site. Hence, the question arises whether in general GU pairs are able to create divalent metal ion binding sites due to their distinct molecular geometry or whether other (additional) structural or molecular prerequisites have to be obeyed for this purpose. The special role of GU pairs with regard to Mg^{2+} binding is further suggested by the finding that yeast $tRNA^{Phe}$, too, contains a G4-U69 base pair in the acceptor stem not far from the Mg^{2+} binding site [3].

Thus it appeared interesting to address this question by studying various acceptor stem helices derived from different tRNAs with respect to their Mg^{2+} binding behaviour by means of proton NMR spectroscopy using Mn^{2+} as a probe since it is well-established that manganese can substitute for magnesium in many biological systems without significantly hampering their function [7]. A similar approach using paramagnetic ions that permitted detection of divalent metal ion binding sites in whole tRNA molecules was used, e.g., already by Chao and Kearns [8] and Hurd *et al.* [9]. However, though there is much evidence of divalent metal ion binding in tRNA (see, e.g. [1, 3] and references therein) and other RNA molecules possessing a pronounced three-dimensional folding, e.g., ribozymes [10, 11], to the best of our knowledge, no studies describing the existence of specific divalent metal ion binding sites in relatively short oligoribonucleotide duplexes have been published.

MATERIALS AND METHODS

Oligoribonucleotide synthesis

The oligoribonucleotides were chemically synthesized by the H-phosphonate method on a Gene Assembler Plus (Pharmacia) as described previously [12, 13]. The 5′ and 2′ OH groups of ribose were protected by 4,4′-dimethoxytrityl and *tert*-butyldimethylsilyl protecting groups, respectively. Protection of the exocyclic amines in adenine and guanine was achieved by dimethylamino-methylene protection groups [7,8]. The purification of the oligonucleotides was performed by HPLC on a Vydac C_4 column. The RNA synthones were purchased from DIAGEN (Düsseldorf, Germany). The purity of the samples was checked by 20% PAGE in the presence of 7 M urea.

Sample preparation

NMR samples contained between ca. 1.7 mg and 13 mg of RNA in 0.5 ml H_2O/D_2O (9:1, v/v) buffer solution with 100 mM NaCl, 10 mM sodium phosphate, pH 6.5, and small amounts of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal chemical shift reference. The RNA concentrations in the NMR sample correspondingly were between about 0.6 mM and 4 mM. Depending on RNA concentration, $MgCl_2$ has been added to the NMR samples to give Mg^{2+} concentrations between 5 mM and

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15 mM. $MnCl_2$ concentrations in each case were less by a factor of exactly 1000, yielding values between 5 μM and 15 μM.

Before measuring, all samples have been annealed by heating them to 80°C for 5 min and subsequently slowly cooling to room temperature.

NMR spectroscopy

All proton NMR spectra were recorded on an AM 500 spectrometer (Bruker, Karlsruhe, Germany) at a resonance frequency of 500 MHz. The strong water signal was suppressed by the 1−3−3−1 pulse sequence according to Hore [14]. To improve the signal-to-noise ratio, all spectra have been convoluted by a Lorentzian line of half width 2 Hz. The chemical shifts have been referenced to the methyl resonance of internal DSS.

RESULTS

As has been described before [4] the microhelix corresponding to the acceptor arm of $tRNA^{Ala}$ from *E.coli* with seven base pairs and a single-stranded 3'-ACCA terminus (see Fig. 1a; denoted by 18mer/GU in the following) gives rise to well-resolved imino proton peaks which indicate correct base pairing. All of these imino resonances could be uniquely assigned to the individual base pairs on the basis of nuclear Overhauser (NOE) experiments and analyses of the spectra in dependence on temperature. However, the base pair opening rate and correspondingly the imino proton exchange rate of the terminal AU base pair 7 even at fairly low temperatures is so large that its resonance becomes too broad to be detectable (a feature that is common to many duplex-terminating base pairs and especially to all of the base pairs 7 of the duplexes studied in the present work). For this acceptor stem duplex as well as for a variant with the same sequence lacking the single-strand ACCA end (termed 14mer/GU for short in the following), specific binding of Mn^{2+}(Mg^{2+}) between the second and third base pairs could be clearly demonstrated by a distinct broadening of the corresponding imino proton lines upon addition of Mn^{2+} at a ratio of 1:1000 to Mg^{2+} to the sample solution. If a paramagnetic ion like manganese is bound in the vicinity of a proton (i.e. within a radius of about 1 nm) it accelerates the relaxation of the proton spin drastically, giving rise to a broadened NMR line. The extent of the linewidth increase varies as r^{-6} (r—distance between proton and paramagnetic ion) [7]. From the fact that there is only one uniform set of spectra with certain specifically broadened peaks it must be concluded that there is a rapid exchange (on the NMR timescale) between bound and free Mn^{2+} ions. If the Mn^{2+} ions were tightly fixed to their binding sites on the RNA molecules (with residence times of, e.g., seconds or more) only a small fraction of all the RNA molecules in the sample should give rise to specifically broadened lines whereas the vast majority remains totally unperturbed and would yield the 'normal' spectrum since the ratio of Mn^{2+} ions to RNA molecules amounts to ca. 1:150−200. The measured spectra, however, indicate that all RNA molecules are equally influenced on the average, suggesting a fast exchange of Mn^{2+} between bound and free states, and associated with this between different RNA molecules, thereby replacing each time probably a magnesium ion.

Since in the case of the 18mer/GU and the 14mer/GU the postulated binding site was located in the vicinity of base pairs 2 and 3 (probably near the phosphate groups), i.e. close to the identity element base pair G3-U70, the question emerged if the wobble GU pair and the structural modifications possibly associated with it [15] play a role for the creation of specific Mg^{2+}/Mn^{2+} binding sites. Hence, different sequence variants have been investigated with GU being replaced by GC, IC, and IU, respectively.

The sequences of the different duplexes analyzed with respect to their Mn^{2+} binding behaviour are compiled in Fig. 1.

Since the importance of the G3-U70 base pairs was of special interest in this study, it appeared quite natural to replace U70 by a cytidine to produce a regular Watson−Crick GC pair. Thus both the full-length $tRNA^{Ala}$ acceptor arm with a G3-C70 base pair (18mer/GC in the following) and its variant without single-strand 3'-ACCA end (14mer/GC) were synthesized and the proton NMR spectra of these duplexes recorded with and without Mn^{2+}.

The imino proton region of the ^{1}H-NMR spectra of the 18mer/GC before (lower trace) and after addition (upper trace) of Mn^{2+} (7.5 μM) at a temperature of 277 K are shown in Fig. 2. At this temperature the binding of Mg^{2+}/Mn^{2+} is tighter (and probably more specific) than at elevated temperatures. In all cases where a specific ion binding could be detected, the line broadening effects could also be observed at higher temperatures (e.g. 298 K) though distinctly less pronounced which is mainly due to the increased mobility of the ions.

As can be seen in Fig. 2 mainly the lines originating from the imino protons of base pairs 2, 3, and 1 (in that order) are affected (see also Table 1 for a quantitative evaluation), i.e. exactly the same base pairs that were influenced in the wildtype 18mer/GU.

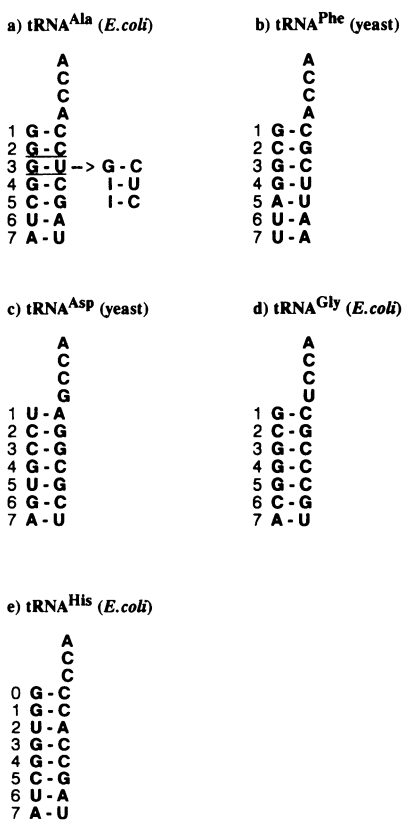

Figure 1. tRNA acceptor stem duplexes used in the proton NMR investigations of Mg^{2+}/Mn^{2+} binding.

Quite similar effects are found with the 14mer/GC though in that case the absolute values of the linewidth increases are distinctly smaller than for the 18mer/GC. Comparison of the line broadening effects for the 18mer/GC quantitatively with those obtained for the wildtype 18mer/GU and 14mer/GU, respectively,(cf. Table 1) reveals a remarkable agreement.

In Table 1 the linewidth changes upon Mn^{2+} addition are given for the $tRNA^{Ala}$ acceptor stem variants with I3-U70 (denoted by 18mer/IU) and I3-C70 (without 3'-ACCA end; 14mer/IC), respectively. In both cases no distinct specific broadening effects could be observed though in the case of the 18mer/IU certain increases for the imino peak linewidths due to base pairs 1, 2, and 3 (U) are observed, which, however, are considerably less than for the GU and GC variants, respectively. This is somewhat surprising since I and G differ from each other by only one amino group at the C2 of the purine ring which is lacking in inosine. However, this amino group is not involved in the hydrogen bonding of the base pair.

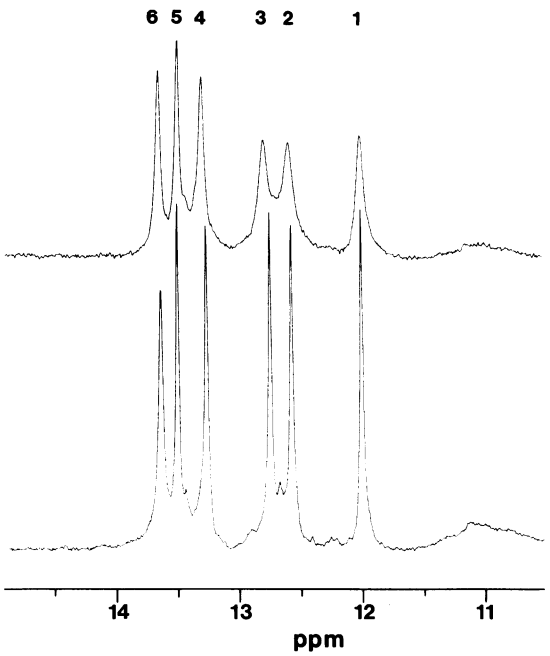

Figure 2. Imino proton region of the ^{1}H-NMR spectra of the 18mer/GC duplex variant of the $tRNA^{Ala}$ acceptor arm before (lower trace) and after (upper trace) addition of 7.5 μM $MnCl_2$ (duplex concentration 1.4 mM, 7.5 mM $MgCl_2$) at 277 K. The numbers refer to the base pair position as indicated in Fig. 1a.

Another tRNA that possesses a GU pair in its acceptor arm at position 4 (G4-U69) is $tRNA^{Phe}$ from yeast (see Fig. 1b). The effect of Mn^{2+} on the acceptor stem duplex as reflected in the imino proton region of the proton NMR spectra is displayed in Fig. 3. Obviously here all the signals originating from the imino protons of base pairs 3, 4(U), and 2 are considerably broadened, whereas the resonance of the G4 imino proton is clearly much less affected than that of the base-paired U69. In absolute values the linewidth increases are in the order of the appearance of the imino signals from low to high field (the base pair number is given in parentheses): 5.4 Hz (6), 9.2 Hz (5), 36 Hz (2), 7.3 Hz (1), 52 Hz (3), 44 Hz (4/U), 13.4 Hz (4/G). With respect to the relative position of the GU pair, and—still more astonishing—even to the absolute linewidth increases this is in accordance with the results obtained for the $tRNA^{Ala}$ acceptor stems (both 18mer and 14mer) since the base pairs most strongly influenced are shifted by just one step in the sequence towards the duplex end, as the position of the GU pair in $tRNA^{Phe}$ is in comparison to $tRNA^{Ala}$.

Another acceptor stem with a GU pair (at position 5) is that of the $tRNA^{Asp}$ from yeast (see Fig. 1c). Similar to the $tRNA^{Ala}$ acceptor arm duplex the microhelix derived from yeast $tRNA^{Asp}$ is recognized by its aminoacyl-tRNA synthetase and specifically aminoacylated with aspartate (J.Rudinger, private communication). The imino proton regions of the proton NMR spectra obtained with this duplex are presented in Fig. 4. Generally the effects of Mn^{2+} addition are much less pronounced than, e.g.,

Table 1. Line broadening (difference of half widths before and after addition of Mn^{2+} ions) of the imino resonances of several acceptor arm duplexes derived from *E.coli* $tRNA^{Ala}$ at a temperature of 277K

Duplex (RNA/$MnCl_2$ concentr.)	line width change (in Hz) upon Mn^{2+} addition for the imino proton base pair 6	4/5	2	1	3(U)	3(G/I)
14mer/GU (1.75 mM/7.5 μM)	9.3	3.6	51	16.2	38	10.4
18mer/GU (0.95mM/5 μM)	4.7	7.6	43	25		14.5
18mer/GC (1.6 mM/7.5 μM)	11	15.3/6.8	45	23	–	39
18mer/IU (4 mM/15 μM)	3.4	6.8	16	15	14	6.1
14mer/IC (0.6 mM/5 μM)	3.6	9.2/10.7	n.d.	n.d.	–	4.2

n.d. not determinable

The arrangement of the columns in the table corresponds to the appearance of the imino resonances for the 18mer/GU from low to high field. If not indicated, the lines due to base pairs 4 and 5 are superimposed and the linewidth change is given for the sum peak. In the case of the 18mer/GU the peaks due to basepair 1 and the U imino proton of basepair 3 are collapsing, and hence the broadening of the sum peak is given. The 14mer/GC (RNA concentration 1.4 mM, 7.5 μM $MnCl_2$) has not been included due to the overlap of the resonances of base pairs 2 and 3 and the appearance of base pair 1 peak at the most upfield position. The corresponding linewidth increases (in Hz) at 282K are (base pair number in parentheses): 3.2 Hz (6), 4.8 Hz (5), 10.1 Hz (4), 26 (3/2), 18 (1). The average uncertainty in the linewdth determination is less that 1 Hz, giving rise to a maximum uncertainty for the linewidth change of less than ± 2 Hz.

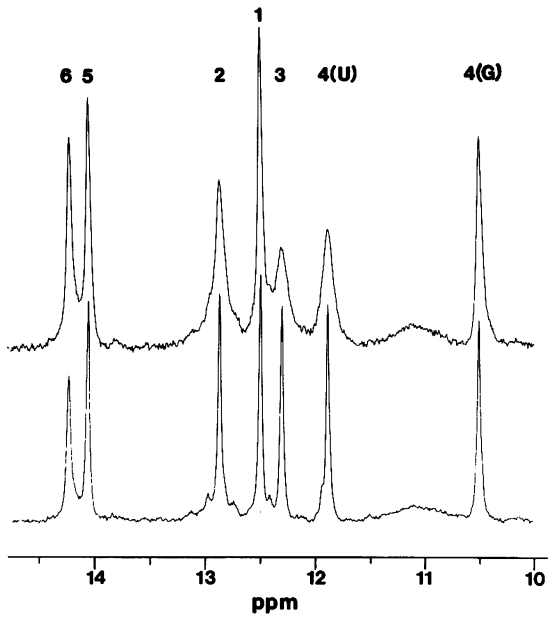

Figure 3. Imino region of the proton NMR spectra of the acceptor arm duplex derived from yeast $tRNA^{Phe}$ before (lower trace) and after (upper trace) addition of 7.5 μM $MnCl_2$ (RNA concentration 1.65 mM, 7.5 mM $MgCl_2$) at 277 K. Base pair numbering according to Fig. 1b.

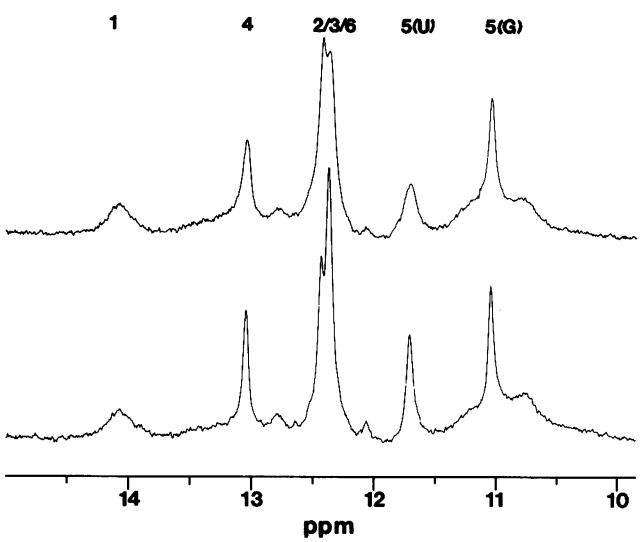

Figure 4. Imino proton resonances of the acceptor stem duplex of yeast $tRNA^{Asp}$ before (lower trace) and after (upper trace) $MnCl_2$ addition (7.5 μM) at 277 K. Base pairs denoted according to Fig. 1c.

for the $tRNA^{Ala}$ or $tRNA^{Phe}$ acceptor stems. Nevertheless, a broadening is found for the U imino proton line of the GU base pair 5 (30 Hz) and the base pair 4 imino signal (15 Hz), whereas the G imino proton peak of base pair 5 (linewidth increase 4 Hz) as well as base pair 1 imino proton resonance (linewidth decrease by ca. 20 Hz ; note that the linewidth of more than 130 Hz is already fairly large) remain essentially unchanged. Due to overlap of the resonance lines of base pairs 2, 3, and 6 no safe assertions can be made about their specific broadening, however, from the appearance there seems to be no significant effect. Again there

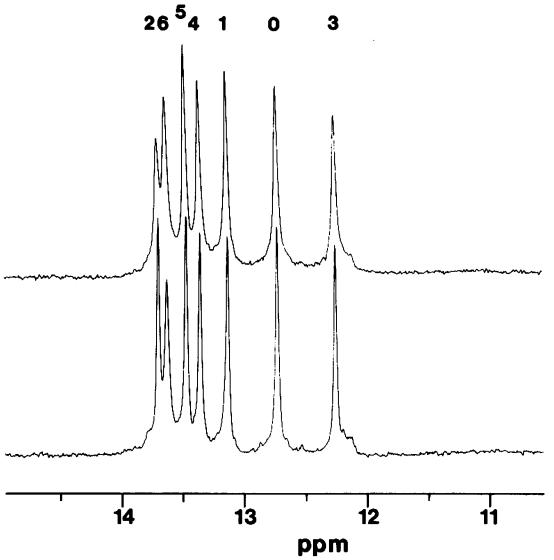

Figure 5. Imino proton resonances of the acceptor stem duplex derived from *E.coli* $tRNA^{His}$ before (lower trace) and after (upper trace) addition of 5 μM $MnCl_2$ at 277 K. RNA concentration 0.84 mM, 5 mM $MgCl_2$. Base pair notation according to Fig. 1e.

is a distinct broadening of the U imino proton resonance in the GU base pair and nearly no effect on the imino peak of the base-paired G.

Finally, two examples of tRNA acceptor stem duplexes are given for which no distinct effects of Mn^{2+} addition on proton NMR linewidths could be detected. The spectra of the acceptor arm duplex of $tRNA^{His}$ from *E.coli* are shown in Fig. 5. The quantitative values for the linewidth changes after Mn^{2+}addition at 277 K are as follows (ordered according to the appearance of the imino signals from low to high field, base pair numbering as in Fig. 1e given in parentheses): 13.6 Hz (2), 0.5 Hz (6), 3.1 Hz (5), 4.2 Hz (1), 4.9 Hz (0), 7.2 Hz (3).

A very similar behaviour with regard to Mn^{2+} binding, i.e., no noticeable line broadening upon Mn^{2+} addition, is likewise found for the acceptor stem duplex derived from *E.coli* $tRNA^{Gly}$ (Fig. 1d; the imino regions of the proton NMR spectra are displayed in Fig. 6). Here the linewidth variations of the imino resonances induced by Mn^{2+} are (base pair numbers given in parentheses) 5.0 Hz (5), 2.1 Hz (1), 6.4 Hz (2), −0.8 Hz (6/4—overlapping lines), 12.1 Hz (3).

DISCUSSION

It has been demonstrated by studying the line broadening effects of small amounts of paramagnetic Mn^{2+} ions (5−15 μM) on the imino resonances of several tRNA-derived duplexes that even relatively short (seven base pairs) acceptor stem helices are able to specifically bind divalent metal ions. However, even in the cases were such specific binding has been found, the ions are not totally immobilized at the oligonucleotide. Rather they rapidly exchange between the tRNA-bound state and the free solution (mean residence times at the RNA molecule being shorter than about 10^{-3}s), as well as between different RNA molecules. Hence, the observed binding sites could be characterized more accurately as preferred residence sites.

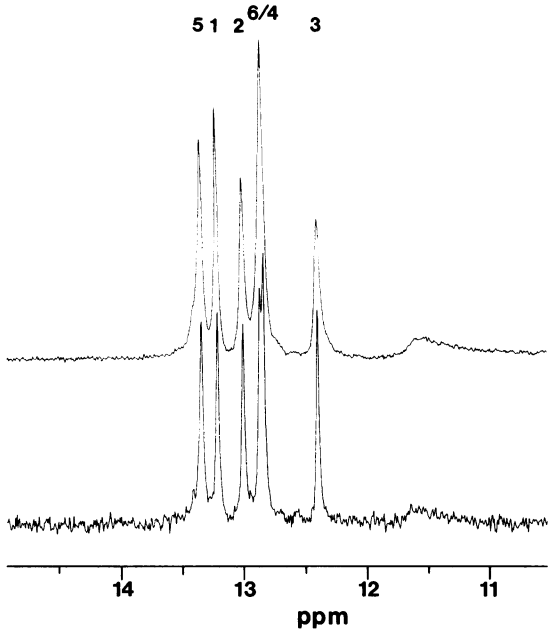

Figure 6. Imino resonance region of the proton NMR spectra of the $tRNA^{Gly}$ (*E.coli*) acceptor stem before (lower trace) and after (upper trace) addition of 5 μM $MnCl_2$ (RNA concentration of 0.7 mM, 5 mM $MgCl_2$) at 277 K. Base pair numbering as given in Fig. 1d.

From the observation of such binding sites near GU wobble base pairs in duplexes corresponding to the acceptor arms of $tRNA^{Ala}$ from *E.coli* [4] and yeast $tRNA^{Phe}$ (present work, see also [3]), one could be tempted to postulate a crucial role of GU pairs for the formation of specific metal ion binding sites, possibly associated with specific modifications of RNA helix geometry induced by this mismatch pair as suggested by van Knippenberg *et al.* [15]. However, a nearly identical Mn^{2+} binding mode was also found for a duplex variant of the $tRNA^{Ala}$ acceptor arm where the GU was replaced by a regular GC pair. In this case, obviously the particular sequence stretch of four guanosines, and the resulting special implications for the stacking behaviour could be of great importance for the local structure as has been pointed out before by Moras *et al.* [16]. Nevertheless, the existence of the GU pair could possibly favour the creation of divalent metal ion binding sites, presumably by the involvement of the C2-amino group of G which does not directly participate in base pair hydrogen bonding. Removing this NH_2 group by replacing guanosine for inosine clearly diminishes the strength and the specificity of Mn^{2+} binding. It seems noteworthy in this context that a $tRNA^{Ala}$ acceptor stem microhelix with an I3-U70 base pair cannot be aminoacylated with alanine by the $tRNA^{Ala}$ synthetase in contrast to the wildtype (G3-U70) microhelix [17]. Apparently both the structural variations induced by GU as compared to GC (unpublished results) and the free 2-amino group are essential for the aminoacyl-tRNA synthetase recognition since the G3-C70 microhelix is likewise not aminoacylated [17]. This assumption is also corroborated by the lack of specific Mn^{2+} binding sites for the $tRNA^{Gly}$ acceptor stem although in this case a stretch of three guanosines is present, however, without a GU pair.

The supposition of a particular potential of GU pairs for the creation of specific divalent metal ion binding sites is further supported by the observation of a Mn^{2+} effect on the imino resonances of the basepairs 4 and 5 (U5-G68) in yeast $tRNA^{Asp}$ acceptor stem duplex, though the UG pair there has an environment which differs clearly from that in $tRNA^{Ala}$ and $tRNA^{Phe}$ acceptor stems. In $tRNA^{Asp}$ the UG pair is sandwiched between two purine–pyrimidine basepairs in a sequence $^{5'}$purine–U68–purine$^{3'}$, whereas in the case of *E.coli* $tRNA^{Ala}$ and yeast $tRNA^{Phe}$ a three-purines stretch is found: $^{5'}$purine–G3(4)–purine$^{3'}$, where the central G is forming a wobble GU pair, giving rise to a markedly deviating stacking [16]. In the $tRNA^{Asp}$ acceptor duplex the effect of Mn^{2+} on line broadening is not as pronounced as for $tRNA^{Ala}$ and $tRNA^{Phe}$ duplexes, respectively, which again could possibly be associated with the above-mentioned different stacking patterns due to the varying sequence contexts.

Evidently, the presence of the single-stranded 3'-terminus does not markedly affect the Mn^{2+} binding behaviour although for the 14mer/GC the effects appear to be less pronounced than in the corresponding full-length acceptor stem (18mer/GC). Perhaps here the stabilizing effect of the ACCA end [4] favourably influences the binding capability.

An interesting feature of the binding studies with duplexes containing GU pairs is the observation that the linewidth increase following Mn^{2+} addition is always distinctly greater for the U imino proton resonance than for the G imino peak. Investigations of the imino proton exchange by analyzing the imino resonance linewidths in dependence on temperature revealed that the activation enthalpy for the imino proton exchange (which can be regarded as a measure of base pair stability [4]) was in all cases lower for the U imino protons within a GU (or IU) pair than for the G (or I) imino protons (e.g., 191 kJ/Mol and 236 kJ/Mol for U and G, respectively, for the 18mer/GU; unpublished results). It remains yet to be clarified if there exists an interrelation between these phenomena.

In general, it is not clear if there is a functional role for the observed metal ion binding sites. Moreover, in the intact tRNA the binding sites found in this study could be altered by interaction with the neighbouring parts of the tRNA molecule. As early as 1974, Jones and Kearns [18] derived from proton NMR investigations using a paramagnetic rare earth ion (Eu^{3+}) as a shift reagent, a metal ion binding site near the U6-A67 base pair just in the corner of the L of yeast $tRNA^{Phe}$. In *E.coli* $tRNA^{Ala}$ the Mg^{2+} ion bound near the identity element suggests a possible role in tRNA synthetase recognition. Perhaps even in the other cases where a more or less specific metal ion binding was observed this could be of importance for specific and correct interactions with other macromolecules. Moreover, it should be noted that there can be differences in the binding models of Mg^{2+} and Mn^{2+} ions, since Mn^{2+} is prone to bind with both nitrogen and oxygen and hence could be associated with the purine N7 nitrogens, whereas Mg^{2+} prefers oxygen ligands [11].

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