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The Role of Metal Ions in RNA Biochemistry

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Approximately two-thirds of the elements in the periodic table can be categorized as metals. Besides luster, malleability, and conductivity, one of the fundamental characteristics of metals is their low ionization potential. As a result, the ionic forms of these elements predominate in the biosphere. Considering the diverse properties of these ions, it is not surprising that through the process of evolution, metal ions have been co-opted into numerous roles in biology. Metal ions are required for so many biochemical reactions that it is likely that they also had an important role in the RNA world. To understand both modern and prebiotic RNA biochemistry, it is therefore essential to have a basic understanding of these inorganic elements.

Metal ions were abundant in the primordial soup. It is believed that 3.8×10^9 years ago, the ocean was between 80°C and 100°C with a pH possibly as low as 6 (Bengston 1994). Table 1 shows the concentrations of the most common metal ions in today's seas and in blood plasma. Although the concentrations of most of these ions in the prebiotic ocean are not known, the higher temperature and lower pH relative to the current ocean would have solvated a variety of ions and leached metal ions from the mineral-rich ocean beds. Therefore, the concentrations would have been significantly higher than the current values. One important additional difference is the extremely low concentration of easily oxidized metal ions such as Fe(II). Ferrous ion has been predicted to have been very abundant in the primordial ocean, as high as 0.1 mM (Bengston 1994). Interestingly, this change is probably due to the fact that early life forms produced molecular oxygen from photosynthetic reactions that in turn caused a cascade of oxidation reactions to occur. The conversion of Fe(II) to the more acidic Fe(III) resulted in the formation of oxyhydroxide polymers that precipitated out of the ancient oceans, depleting the ion

Table 1 Metal ion abundance in the modern oceans and biological fluids

Ionic species	Modern ocean (mM) ^a	Blood plasma (mM) ^a	In mammalian cells ^b	Mammalian extracellular fluid ^b
Na ⁺	470	138	10	145
K ⁺	10	4	140	5
Mg ⁽⁺⁺⁾	50	1	30	1
Ca ⁽⁺⁺⁾	10	3	1	4
Fe ⁽⁺⁺⁾	1 × 10 ⁻⁴	2 × 10 ⁻²		
Zn ⁽⁺⁺⁾	1 × 10 ⁻⁴	2 × 10 ⁻²		
Cu ⁽⁺⁺⁾	1 × 10 ⁻³	1.5 × 10 ⁻²		
Co ⁽⁺⁺⁾	3.1 × 10 ⁻⁶	2 × 10 ⁻³		
Ni ⁽⁺⁺⁾	1 × 10 ⁻⁶	0		

^aData from Pan et al. (1993).^bData from Cowan (1995).

from the biosphere and giving the banded iron formations observed in sedimentary rocks. With respect to modern biochemical studies, this shift in metal ion availability justifies studying what are today considered “non-physiologically relevant” metal ions in RNA catalysis and structure in order to probe events related to the early RNA world. The more reactive metal ions may in fact have helped to drive the evolution from the RNA world to the modern protein world, where the polymer backbone is more stable to the potential side reactions induced by the metal cofactors.

A variety of physical properties can be used to characterize the behavior of metal ions. A number of textbooks have excellent discussions of metal ion behavior (Huheey 1983; Cotton and Wilkinson 1988; Cowan 1995; Richens 1997) and should be consulted for more detailed descriptions. Table 2 lists ionic radii, pK_a values (of the aqua ions), hydration numbers, water exchange rates and ΔH_{hyd} for a number of relevant metal ions. It should be remembered when dealing with tables such as these that many of the properties listed are for the most common forms of the ions. Parameters specific to a metal ion complex can have significant effects on the values for other physical properties, especially for the transition metals.

Another useful concept in consideration of metal ions is that of hardness and softness. This property summarizes the general affinity of a Lewis acid (the metal ion) for a Lewis base (the ligand) in a manner independent of the acidity or basicity of the species and reflects the degree of covalency in the metal–ligand bond. The general trend is that hard metal ions preferentially bind hard ligands and vice versa. In RNA, the hardest ligand is the anionic phosphate oxygen. In a recent quantitative description, the absolute hardness (η) is proportional to the difference between I,

the ionization potential, and A , the electron affinity, of the species (Pearson 1988). Absolute softness is defined as η^{-1} . The interested reader should consult Pearson's paper for how η and χ (the absolute electronegativity) are applied quantitatively to any given acid–base reaction. It should be noted, however, that the ligands coordinated to a metal ion will influence its actual hardness or softness when in the context of a specific interaction. As an approximation, the presence of soft ligands in the coordination sphere of a metal tends to make the central ion softer, whereas hard ligands make the ion harder.

With these physical properties in mind, one can address the question of the role metal ions play in RNA biochemistry. Since each residue contains an anionic phosphodiester group, the principles of charge neutralization and electrostatic condensation dictate that cations must be closely associated with the polyanionic RNA molecule (Record et al. 1978; Manning 1979; Anderson and Record 1995). In principle, these can be any cationic species, but in general, the condensation layer consists of the abundant surrounding monovalent and divalent ions. In vivo, Mg(II) and K^+ are believed to dominate in this role. Charge neutralization becomes particularly important during the process of RNA folding, as the negatively charged backbones from two or more regions of the primary sequence most come close together in space. Without cations to screen these charges, the repulsive forces generated in the close-packed structure would overwhelm the energetically favorable interactions that dictate the proper three-dimensional structure. Since there is a formal charge of -1 for every residue, RNAs carry around a sufficient number of metal ions in a condensation layer to neutralize the charge. Studies measuring the number of Mg(II) ions bound to different RNAs have borne out this expectation (Table 3). The majority of these metal ions bind the RNA nonspecifically, solely dictated by electrostatic considerations. Each individual counterion is very weakly bound and in rapid exchange with more freely diffusing ions. Furthermore, they cannot be localized by most biophysical techniques because of the diversity of binding environments at any given instant.

Among the metal ions that bind an RNA, a subset interact specifically. Metal ions generally bind to these sites more tightly than to the nonspecific ones. They are better localized because of discrete interactions and they cannot be as easily substituted by other ions (Laing et al. 1994; Gluick et al. 1997). For that reason, these sites dictate the metal preferences of the RNA molecule as a whole. These specific sites can be further subdivided based on the role of the metal ion in the biochemistry. Metal ions can serve in structural roles, or as catalytic cofactors or potentially as

Table 2 Properties of selected metal ions

Metal	Oxidation state	Hydration number ^a	Ionic radius (Å) ^{a,b}	ΔH_{hyd} (kJ/mole) ^a	Water exchange rate (s ⁻¹) ^a	pKa of aqua ion ^a	Absolute hardness η^c	Absolute electro-negativity χ^c
Group 1A								
Li	1+	4	0.59	-514.1	$\sim 10^9$	13.8 ^c	35.12	40.52
Na	1+	6	1.02	-405.4	$\sim 10^9$ ^d	14.48 ^e	26.21	21.08
K	1+	6	1.38	-320.9	$\sim 10^9$ ^d		17.99	13.64
Group 2A								
Mg	2+	6	0.72	-1922.1	$\sim 10^6$	11.42 ^e	47.59	32.55
Ca	2+	8	1.12	-1592.4	$\sim 10^8$	12.70 ^e	19.52	31.39
Sr	2+	6	1.18	-1444.7	$\sim 10^9$ ^d	13.18 ^e	27.3	16.3
Ba	2+	6	1.35	-1303.7	$\sim 10^9$	13.82 ^e		
1st row transition metals								
Cr	2+	6	0.80	-1849.7	$\sim 10^9$		7.23	23.73
Cr	3+	6	0.62	-4401.6	2.4×10^{-6}	4.00	9.1	40.0
Mn	2+	6	0.67	-1845.6	2.1×10^7	10.6	9.02	24.66
Fe	2+	hs 6	0.78	-1920.0	4.4×10^6	9.5	7.24	23.42
Fe	3+	ls 6	0.61	-4376.5	$\sim 10^{-3}$	0.70	12.08	42.73
Co	2+	hs 6	0.65	-2054.3	3.2×10^6	2.19 ^e	8.22	25.28
Co	3+	ls 6	0.55		$\sim 10^{-1e}$	9.65	8.9	42.4
Ni	2+	hs 6	0.61	-2105.8	3.4×10^4	9.86	8.50	26.67
		ls 6	0.55					
		T _d 4	0.55					
		sq 4	0.66					

(continues)

Table 2 (Continued)

Metal	Oxidation state	Hydration number ^a	Ionic radius (Å) ^{a,b}	ΔH_{hyd} (kJ/mole) ^a	Water exchange rate (s ⁻¹) ^a	pKa of aqua ion ^a	Absolute hardness η^c	Absolute electro-negativity χ^c
Cu	1+	6	0.77	-594.1			6.28	14.01
Cu	2+	6	0.73	-2100.4	4.4×10^9	7.97	8.27	28.56
Zn	2+	6	0.74	-2044.3	$\sim 10^7$	8.96 9.60 ^e	10.88	28.84
2nd row transition metals								
Ru	3+	6	0.68		1.8×10^{-2}		10.7	39.2
Pd	2+	4	0.64		5.6×10^2	2.3	6.75	26.18
Cd	2+	6	0.95	-2384.9	$\sim 10^8$	10.08 11.7 ^e	10.29	27.20
3rd row transition metals								
Pt	2+	4	0.60		3.9×10^{-4}	~ 2.5	8.0	27.2
Hg	2+	6	1.02	-1853.5	$\sim 10^9$	3.4	7.7	26.5
Miscellaneous								
Pb ^f	2+	6	1.19	-1479.9		7.8	8.46	23.49
Tl	1+	6	1.50	-325.9		13.2	7.16	13.27

^aData from Richens (1997).^bIonic radii are listed for the appropriate coordination number. Other coordination numbers are known for most of these ions and have different effective radii. Species with higher coordination numbers in general have larger ionic radii.^cData taken from Pearson (1988). χ and η parameters for a number of potential ligands are also tabulated in this reference, however, exact data on biological ligands are not currently available.^dData from Lincoln and Merbach (1995).^eData from Huheey (1983).^fThere is no evidence for a monomeric aqua ion of Pb(II). It is observed as an oligomer in aqueous solution (Richens 1997).

Table 3 Mg(II) binding and uptake studies on various RNAs

RNA	Method	Conditions	Length (nt)	Metal ions	Nr/metal ion	K_d (M)		Reference
						strong sites (# of sites)	weak sites (# of sites)	
<i>E. coli</i> tRNA ^{Met}	equilibrium dialysis	0.17 M Na ⁺ , pH 7.0, 4°C	76	27	2.8	3.4×10^{-5} (1)	2.4×10^{-3} (26)	Stein and Crothers (1976)
<i>E. coli</i> tRNA ^{Glu}	equilibrium dialysis	0.10 M Na ⁺ , pH 7.0, 4°C	76	37	2.1	1.3×10^{-5} (1)	1.2×10^{-3} (36)	Bina-Stein and Stein (1976)
Yeast tRNA ^{Phe}	fluorescence titration	0.032 M Na ⁺ , pH 6.0, 10°C	76	23.5 ± 8	3.2	1.1×10^{-5} (6.5 ± 3)	1.7×10^{-4} (17 ± 5)	Römer and Hach (1975)
Yeast tRNA ^{Phe}	calorimetry	0.01 M Na ⁺ , pH 7.2	76	24	3.2	1.0×10^{-6} (4)	9.1×10^{-5} (20)	Rialdi et al. (1972)
Yeast tRNA ^{Phe}	²⁵ Mg NMR	0.17 M Na ⁺ , pH 7.0, 25°C	76	53 ± 8	1.4	<10 ⁻⁴ (3-4)	4.5×10^{-3} (50 ± 8)	Reid and Cowan (1990)
<i>B. subtilis</i> RNase P	gel filtration	0.1 M NH ₄ ⁺ , pH 8.0, 37°C	400	90 - 130	~4		$(0.39 \pm 0.08) \times 10^{-3}$ (95 ± 6)	Beebe et al. (1996)
Hammerhead ribozyme 16	fluorescence titration	0.40 M Li ⁺ , pH 6.0, 25°C	55	29 ± 5	1.9	n.d.	n.d.	A. Feig and O. Uhlenbeck (unpubl.)

n.d. indicates not determined.

both simultaneously. To date, the designation of a site as functional generally has been related to the spatial proximity of the ion to the catalytic center rather than direct experimental evidence that the ion actively participates in the catalytic event.

The specificity of a binding pocket, defined as the relative affinity of different metal ions for the site, can result from a variety of factors, including the hardness of an ion, the identity of the coordinating ligands, the ionic radius, the preferred coordination geometry of the ion, and the metal's hydration number. Very few metal-binding sites have been probed sufficiently to fully define their specificities (Bukhman and Draper 1997; A.L. Feig et al., in prep.). The main problem is that to probe specificity, the site under consideration must remain independent even when ions bind at other places on the RNA. The site must also be uniquely identified by the biophysical technique being used. The specific metal ion interactions among catalytic RNAs are the best defined because the enzymatic activity can be used as a probe (Table 4). The metal ion specificities for these RNAs are composite parameters, however, simultaneously reflecting all of the binding sites required for activity. Whereas some of the ribozymes require a specific metal cofactor, others have less strict requirements. The larger RNAs tend to be more specific than the smaller ribozyme species. This increased specificity makes sense. Since the overall fold is more complicated, there are greater opportunities for specific interactions and there are more tertiary contacts that must be maintained by these ions. In addition to specificity, the apparent Michaelis constants for metal ion cofactors ($^{Metal}K_M$) are often used to describe the total contribution of the metal-binding sites on the catalytic activity (Clouet-d'Orval and Uhlenbeck 1996; McConnell et al. 1997). Although this parameter is an important characteristic of a catalytic RNA, it potentially reflects the effects of multiple metal-ion-binding events and may give little information regarding the properties of any individual metal-binding site.

There are several substantial experimental challenges for the RNA bioinorganic chemist. First and foremost, methods must be found that identify the metal ions bound to specific sites and separate them from the bulk ionic condensation events. As discussed below, any single technique is unlikely to locate all such sites, so multiple parallel approaches will almost certainly be required. Once a site has been identified, the next step is to relate it to a discrete property of the RNA. Any given site can be involved in maintaining local or global structure and can potentially participate in catalysis. Another complication is that RNA molecules and their associated metal ions are conformationally dynamic. Specific metal

Table 4 Metal ion specificity of various natural ribozymes

Ribozyme	Functional	Nonfunctional	Reference
hammerhead	Mg(II), Mn(II), Ca(II), Cd(II), Co(II)	Ba(II), Sr(II), [Cr(NH ₃) ₆] ⁺⁺⁺ , Pb(II), Zn(II), Tb(III), Eu(III)	Dahm and Uhlen- beck (1991); A.L. Feig et al. (in prep.)
hairpin	All tested, including [Cr(NH ₃) ₆] ⁺⁺⁺		Hampel and Cowan (1997); Nesbitt et al. (1997); Young et al. (1997)
hepatitis δ virus	Mg(II), Mn(II), Ca(II), Sr(II)	Cd(II), Ba(II), Co(II), Pb(II), Zn(II)	Wu et al. (1989); Suh et al. (1993);
<i>Neurospora</i> VS	Mg(II), Mn(II), Ca(II)		Collins and Olive (1993)
RNase P	Mg(II), Mn(II), Ca(II)	Sr(II), Ba(II), Zn(II), Co(II), Cu(II), Fe(II), Ni(II)	Smith et al. (1992); Smith and Pace (1993)
<i>Tetrahymena</i> Group I	Mg(II), Mn(II)	Ca(II), Sr(II), Ba(II), Zn(II), Co(II), Cu(II)	Grosshans and Cech (1989); McConnell et al. (1997)
Group II	Mg(II)	Ca(II), Mn(II)	Chin and Pyle (1995)

ions generally exchange rapidly and need not be present in the same location in all of the conformational states. Therefore, if one is to accurately define the function of the metal ion, techniques must be available that can study metal–RNA interactions in different time frames. Finally, metal ion interactions may significantly affect RNA folding pathways, and controls should always be included to determine whether the conditions used resulted in the stabilization of an alternate conformation of the RNA (Uhlenbeck 1995).

One important issue addressed by this chapter is how metal-binding sites are identified. One can broadly group the techniques into three categories: (1) use of a biophysical technique (X-ray crystallography, NMR, etc.) with the native RNA and the native metal ions, (2) replacing the

native metal ion with a nonnative ion more sensitive to a biophysical technique, and (3) specific synthetic modifications of the RNA that alter the local metal-binding properties. All three approaches have advantages and disadvantages and thus are best used in combination.

A few dozen specific RNA-binding sites have been identified. A number of the better-studied examples are presented in Table 5. We focus on a few of these sites that show either interesting structures or substantial specificity. We do not discuss the role of some ions in promoting catalysis. That topic is covered in Chapters 11 and 13 of this volume and has been the subject of a number of recent review articles (Pan et al. 1993; Yarus 1993; Smith 1995; McKay 1996; Pyle 1996). Instead, the central issue will be the binding sites themselves. What are the interactions between metal ions and RNA? Why does one site show specificity for a particular metal ion whereas another site does not? Does this specificity derive from the metal ion being used in the experiment, or is it a result of the RNA structure? Finally, we address the issue of whether the experimental approach being used to study a particular question biases the results toward identifying one type of metal-binding site over another.

CRYSTALLOGRAPHICALLY CHARACTERIZED METAL-ION-BINDING SITES

One useful way in which bioinorganic chemists classify metal-binding sites is by their nuclearity, or the number of metal ions that are held together in the structural/functional unit. The common divisions used for proteins, mononuclear, dinuclear, and polynuclear sites, are also suitable for RNA sites. The rationale behind this organization derives from the functional and spectroscopic differences between these classes. Although many fewer RNA sites are available, mono- and dinuclear clusters have both been characterized crystallographically. Polynuclear clusters of ions have also been observed and given names such as “metal zippers” (Correll et al. 1997), but it is still unclear whether the sites are cooperatively linked and thus act as a single element or just represent the clustering of mononuclear sites in a complex folded region of the RNA molecule. As more of these multinuclear motifs become available for detailed study, it will hopefully become clear whether the core ions act individually or as a unit to promote RNA structures.

The majority of the well-characterized metal-binding sites fall into the mononuclear category. These sites include all of the metal-binding sites observed on tRNAs, as well as most of the sites in the hammerhead ribozyme, P4-P6 domain of the *Tetrahymena* group I intron and the 5S rRNA fragment. The mononuclear sites tend to be quite variable with

Table 5 Selected well-characterized metal-ion-binding sites on RNA

RNA	Binding site	Metal ions that bind site	Closest contacts	Methodology ^a	Notes
tRNA	8-12 turn	Mg(II), Sm(III)	O _p -U8 O _p -A9		b-d
	D-stem	Sm(III)	O _p -U7 O _p -A14		b
	D-loop-1	Mg(II), Sm(III)	O _p -A20 O _p -A21		b-d
	D-loop-2	Co(II)	N7-G15		b
	D/TΨC-loop-1	Mg(II), Mn(II), Pb(II)	O _p -G19, N7-G20, O4-U59, N3-C60	Pb(II) cleavage, X-ray	b-d
	D/TΨC-loop-2	Mg(II), Sm(III)	O _p -G57, O _p -A14		b, d
	acceptor arm 1	Mn(II), Mg(II)	G3-U70	NMR	e
	acceptor arm 2	[Co(NH ₃) ₆] ⁺⁺⁺	O4-U69		d
	anti-codon loop-1	Mg(II)	O _p -Y37		c
	anti-codon loop-2	Pt(II)	N7- ^m G34		b
	anti-codon loop-3	Pb(II)	O2- ^m C32 N7-Y37		f
	anti-codon stem	[Co(NH ₃) ₆] ⁺⁺⁺	N7-G42		d
	variable loop-1	Sm(III), Pb(II)	O6-G45 N7-G45		b, f
variable loop-2	Hg(II)	O4-U47		b	
variable loop-3	[Co(NH ₃) ₆] ⁺⁺⁺	O _p -A44		d	
Hammerhead ribozyme	domain II	Mg(II), Mn(II)	O _p -A9	X-ray, phosphorothioate	g, h

uridine turn (G5)	Mg(II), Mn(II), Tb(III)	O6-G5	X-ray, inhibition, electrophoretic mobility	h-j
cleavage site	Mg(II)	O _p -A1.1	X-ray, phosphoro- thioate	i
P5-U·G	Mg(II), [Os(NH ₃) ₆] ⁺⁺⁺	U120·G201 G121·U202		k
P5b-G·U	[Co(NH ₃) ₆] ⁺⁺⁺ , [Os(NH ₃) ₆] ⁺⁺⁺	G147·U156 G148·U155	X-ray, NMR	k, l
P5c	[Os(NH ₃) ₆] ⁺⁺⁺	O4-G174 N7/O6-G175 O _p -G176 O _p -U177 O6-G164		k
3 helix junction (P5a, P5b, P5c) J6/6a	Mg(II)			k
	Mg(II)	C213 A256 G257		k
	[Os(NH ₃) ₆] ⁺⁺⁺			k
A-rich bulge di-Mg(II) site	Mg(II), Sm(III)	O _p -185,186		k
P5a-near A-rich bulge	Mg(II)	O6-G188		k
J5/5a	Mg(II)	O _p -C128		k
J4/5	Mg(II)	O _p -G112		k

(continues)

Table 5 (Continued)

RNA	Binding site	Metal ions that bind site	Closest contacts	Methodology ^a	Notes
5S rRNA	loop E di-Mg(II) site	Mg(II)	O _p -G100 O _p -A101		m
	loop E	Mg(II)	O6-G98 O _p -C97 O _p -A76		m
	stacked GG site	Mg(II)	O6-G105 O6-G106		m
IRE		[Co(NH ₃) ₆] ⁺⁺⁺	U6-G26	NMR	n

^a Data from x-ray crystallographic studies unless otherwise noted.

^b Data from Jack et al. (1977).

^c Data from Holbrook et al. (1977).

^d Data from Hingerty et al. (1982).

^e Data from Ott et al. (1993); Allain and Varani (1995).

^f Data from Brown et al. (1985).

^g Data from Pley et al. (1994).

^h Data from Scott et al. (1995).

ⁱ Data from Scott et al. (1996).

^j Data from A.L. Feig et al. (in prep.).

^k Data from Cate and Doudna (1996).

^l Data from Kieft and Tinoco (1997).

^m Data from Correll et al. (1997).

ⁿ Data from Gdaniec et al. (1998).

respect to their ligand coordination sphere, and almost universally appear in regions of irregular secondary or tertiary structure. The nonbridging phosphate oxygens are the most common non-water ligands found in the inner-coordination sphere of Mg ions in RNA structures. Because they are very hard ligands and the sites of greatest charge density on the RNA, this finding is not unexpected. Other common and somewhat softer ligands include the 2'-hydroxyl groups, the N7 nitrogens of the purine bases, and the keto oxygens of G and U. The only consistent feature in RNA metal-binding sites is that the ions are significantly hydrated. Often only one or two inner-sphere contacts are made between the ion and the RNA to which it is specifically bound, and in some cases, the binding is entirely mediated through outer-sphere contacts. This hydration contrasts with the majority of the metal-binding sites observed in proteins where water ligands tend to occupy very few coordination sites at the metal center (Lippard and Berg 1995).

It remains a challenge in RNA crystallography to unambiguously identify Mg ions, because the electron density is similar to that of hydrated sodium ions or waters. Holbrook et al. (1977) proposed several criteria, including the size and the height of the electron density peaks and the coordination geometry, to distinguish Mg ions from its look-alikes. Hydrated magnesium ions are separated from sodium aqua ions based on the smaller diameter (0.8 Å smaller) of the latter. Another useful approach is to make use of the fact that in many cases, Mn(II) can compete with Mg(II) for specific binding sites. Due to their greater electron density and anomalous scattering properties, Mn(II) ions are often more easily identified in the electron density maps and provide additional support for the assignment of a peak as a hydrated Mg(II) ion (Holbrook and Kim 1997). One must remember, however, that Mg(II) and Mn(II) are not identical. This substitution is often advantageous, but the exact orientation of the metal ion within the overall binding site may shift as a function of this substitution.

Among several well-defined metal-ion-binding sites in tRNA^{Phe} (Fig. 1A), the first example of a mononuclear site is at the intersection of the D-loop and the TΨC-loops and was designated site 1 in the original orthorhombic crystal form (Holbrook et al. 1977) and site 3 in the monoclinic structure (Fig. 1B) (Jack et al. 1977). The closest contact between this ion and the RNA molecule is the 1.9 Å distance to the pro-S_P oxygen of phosphate 19 (Holbrook et al. 1977), which clearly represents an inner-sphere interaction. The remaining contacts to the RNA are 3.5–4.5 Å and thus are probably indicative of outer-sphere interactions mediated by hydrogen bonding of a bound water to the RNA. Although in the best

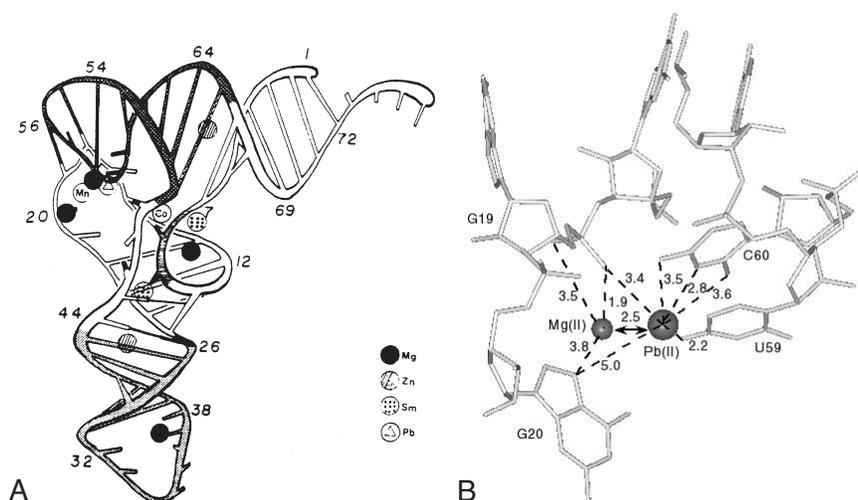


Figure 1 (A) Metal ions found in the crystal structure of yeast tRNA^{Phe} and the relationship between these sites and the overall structure. (Reprinted, with permission, from Pan et al. 1993.) (B) Superposition of the Pb(II) and Mg(II) binding sites from the intersection of the D and TΨC loops. Waters of hydration are not shown and contact distances are given in angstroms.

cases these water molecules can be observed directly in the electron density maps, this site is typical in that the ordering of the water molecules around the metal ion is not resolved.

This tRNA site is useful to illustrate how various ions bind to the same site, since lead and manganese ions have also been characterized crystallographically in this pocket. When Pb(II) binds at this site, the center of the electron density is shifted 2.5 Å with respect to Mg(II). Inner-sphere interactions are observed between the lead and the carbonyl oxygen (O4) of residue U59 (2.2 Å) and the heterocyclic nitrogen (N4) of C60 (2.8 Å), and the ion is positioned much further from p19 (3.4 Å) than was the Mg(II) (Fig. 1B). On the other hand, the position of the Mn(II) is closest to the N7 of G20, but maintains the inner-sphere interaction with the pro-S_P oxygen of p19 in a manner similar to Mg(II) when it occupies this site (Jack et al. 1977). Thus, all three ions occupy the same general site but show distinct coordination preferences. These differences make sense for the three ions. Mg(II) is an extremely hard metal ion, so its position is most strongly influenced by the hard phosphate oxygen. Lead, on the other hand, is much softer. The shift toward the carbonyl oxygen of U59 and ring nitrogen of C60 respects this difference. Furthermore, the larger

size of lead and its greater hydration number allow it to fill the entire gap between the residues C60 and C20. The inner-sphere interactions with C60 and U59 do not preclude the outer-sphere coordination to G20 and p19. Mn(II) is too small to span this gap and thus is held to one side of the pocket by its interactions with the N7 and phosphate oxygen. All three ions are sufficiently close together, however, that their binding is mutually exclusive.

Several mononuclear metal-binding sites have been identified crystallographically on the hammerhead ribozyme (Fig. 2A) (Pley et al. 1994; Scott et al. 1995, 1996; Feig et al. 1998). One of these sites is located adjacent to position G5 and was identified based on the binding of Mn(II) and Tb(III), although a small amount of electron density was observed at this site in the presence of Mg(II) (Fig. 2B). This site is located approximately 10 Å from the scissile phosphate. Like the tRNA^{Phe} site discussed above, the different ions occupy overlapping positions that are about 2.1 Å apart. The closest contact in both cases is to the base-pairing face of G5, with longer contacts to the adenosine at residue 6 and the 2'-OH groups of positions 15.3 and 16.2. All three ions are nearly the same distance from G5 but sit either above or below the plane of the base. The resolutions of these structures are insufficient to localize the water molecules coordinated to these ions. It is important to note that these structures were determined from crystals, grown from a mother liquor containing 1.8 M Li₂SO₄. Because lithium would not be observed in the electron density maps, it is possible that the presence of these ions could perturb the position of another ion nearby such that it would not bind in exactly the same manner as it might in a typical solution study. If there is an inner-sphere contact with the N1 position of G5, it almost certainly implies that metal ion coordination at this site induces a tautomerization, because this nitrogen is protonated in the dominant tautomer. Metal binding at this site has been linked to a structural rearrangement at low Mg(II) concentrations by gel electrophoretic mobility studies (Bassi et al., 1995, 1996). It has also been shown that the binding of Tb(III) to this site results in inhibition of the cleavage reaction (Feig et al. 1998).

A second metal-binding site from the hammerhead ribozyme is located near the phosphate of residue A9 (Fig. 2C). This metal-binding site was identified in both X-ray crystal structures (Pley et al. 1994; Scott et al. 1995), but there is a discrepancy between the two with regard to the details observed. The Mg ion found in the all-RNA structure makes an inner-sphere contact to the pro-S_P oxygen of p9 and outer-sphere interactions with the 2'-OH of G8 (3.8 Å), the N7 of G10.1 (3.9 Å) and the N2 of G12 (4.6 Å). In contrast, when Mn(II) was used to localize the metal-

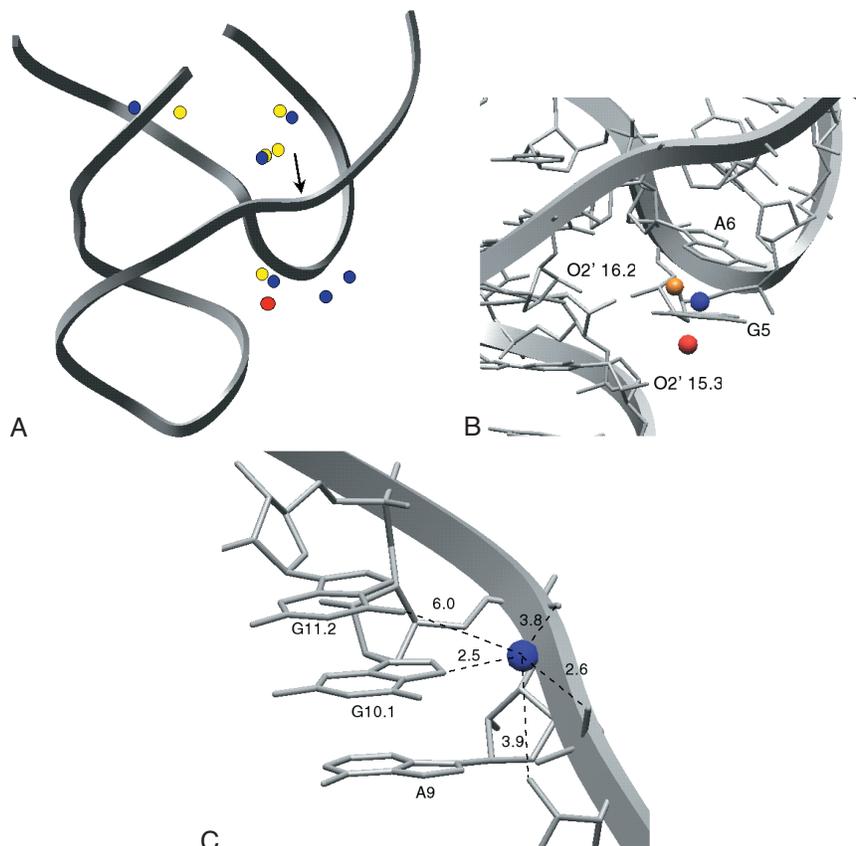


Figure 2 (A) Metal ions found in the crystal structures of the hammerhead ribozyme and the relationship between these sites and the overall structure. The location of the cleavage site is marked by the arrow. Mg(II) is shown in *yellow*, Mn(II) is shown in *blue*, and Tb(III) is shown in *red*. (B) Expanded view of the G5 metal-binding site of the hammerhead ribozyme. Waters of hydration have been omitted. (C) Expanded view of Mn(II) binding to the p9 site of the hammerhead ribozyme. Waters of hydration are not shown and distances are given in angstroms.

binding sites of the RNA/DNA chimeric hammerhead, the metal ion was observed binding directly to the pro- R_P oxygen of p9 as well as to the N7 position of G10.1 (Pley et al. 1994). The different coordination modes, as in the tRNA site discussed above, might result from the metal substitution used to better visualize the ion. Whereas metal binding to this site appears essential for cleavage, the role of this ion in the mechanism is still uncertain (McKay 1996; Peracchi et al. 1997).

The first dinuclear site identified in an RNA molecule is from the P4-6 domain of the group I intron from *Tetrahymena* (Cate and Doudna 1996; Cate et al. 1996, 1997). In total, 12 well-defined Mg(II) ions were identified in the native crystal structure (Fig. 3A). The dimagnesium site from P4-P6 is found as an integral component of the A-rich bulge (Fig. 3B). In this region, the phosphate backbone is highly distorted and the two magnesium ions are found bridged by two phosphate groups. One Mg(II) lies on either side of the backbone with a Mg–Mg separation of 5.3 Å. Each Mg ion makes three inner-sphere contacts with phosphate oxygens. Three unresolved water molecules probably fill out the rest of the coordination sphere. The metal binding helps to hold the backbone in an unusual conformation with a 4.2 Å phosphate-to-phosphate distance between A184 and A186. Residue 185 is involved in reversing the direction of the phosphate backbone. The inner-sphere Mg–O_P distances are unremarkable

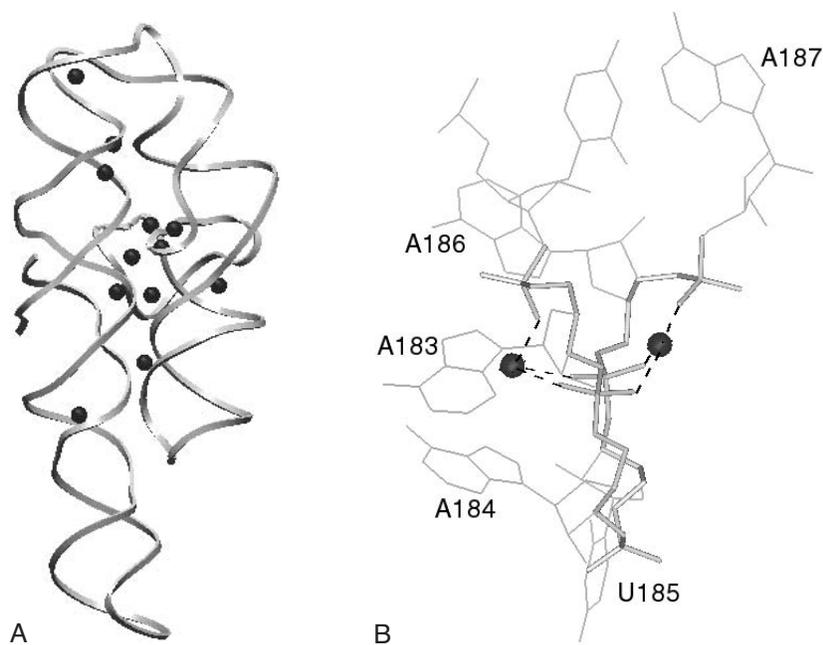


Figure 3 (A) Ribbon diagram of the P4-P6 domain of the group I intron from *Tetrahymena* showing the location of the bound Mg(II) ions. (B) Expanded view of the dimagnesium site from the A-rich bulge of the P4-P6 domain. The Mg(II) ions hold the backbone in a highly distorted conformation by coordination to the phosphate oxygens of A184 and A186. Phosphate oxygens from A183 and A187 also make inner sphere contacts with the Mg(II) ions. The Mg–Mg distance is 5.3 Å. The Mg(II)–O_P distances range from 2.0 to 2.3 Å. Waters of hydration are not shown.

at 2.0–2.3 Å. Both Mg ions lie in the phosphinyl plane of phosphate 186. This orientation has been shown to be less favorable for the binding of metal ions to phosphate oxygens than one in which the metal ion sits 0.9 ± 0.5 Å above or below that plane (Alexander et al. 1990). In the case of phosphate 184, one Mg ion is in-plane, whereas the other rests in the more favorable out-of-plane orientation.

A variety of other metal ions have been soaked into the crystals of P4-P6 and this site will accept Sm(III) ions. Although osmium hexammine and cobalt hexammine are observed to bind in a number of the mononuclear sites on this molecule, neither ion binds efficiently to the A-rich bulge, based on the crystallographic experiments. These two ions are exchange-inert, and the specificity may relate to their inability to make the close contacts to the phosphate oxygens observed in the native structure. Three other Mg ions also bind in this region of the RNA in mononuclear sites and presumably assist in the stabilization of the extensive interhelical packing.

The dinuclear magnesium center observed in the loop E fragment of the 5S rRNA is currently unique among RNA metal-binding sites (Fig. 4A) (Correll et al. 1997). These ions are two of the five that line the major groove of the helical fragment and help pull the backbones to a very narrow 6 Å separation between the phosphate atoms. The high resolution of the structure (1.5 Å) allows the Mg ions to be clearly visualized with their

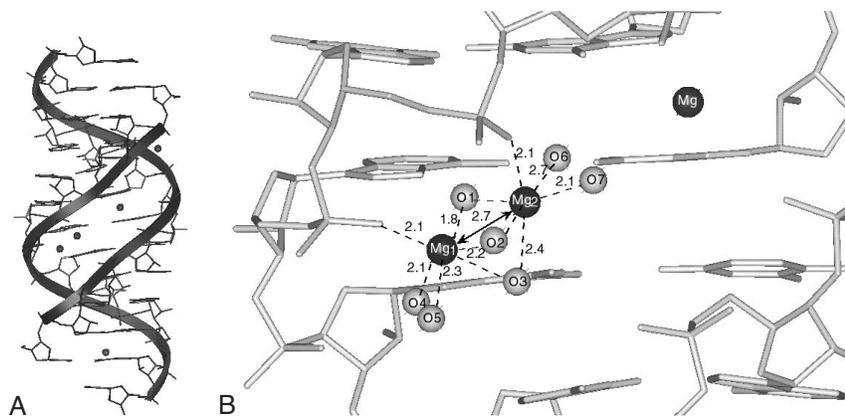


Figure 4 (A) Metal-binding sites from the loop E fragment of the *E. coli* 5S rRNA. (B) Expanded view of the dimagnesium center from the 5S rRNA determined at 1.5 Å resolution. Water molecules directly coordinated to the Mg ions of the dinuclear center are shown, but others have been omitted for clarity. Contact and bond distances are given in angstroms.

coordinated water molecules (Fig. 4B). The magnesiums lie just 2.7 Å apart and have been modeled as being bridged by three water molecules. The protonation state of these waters cannot be determined based on the electron density map, but it is unlikely that they are fully protonated. For the extremely short metal–metal distance, it is likely that at least one and possibly two of the bridging waters are partly or completely deprotonated. In protein and small molecule systems, short metal–metal interactions are often mediated by monatomic bridging ligands (Lippard and Berg 1995; Dismukes 1996; Yachandra et al. 1996). The manganese centers from the photosystem II reaction center and catalase make for good comparisons. The manganese core in PSII consists of a dimer of dimers with a Mn–Mn distance of ~2.7 Å derived from EXAFS and model studies (Yachandra et al. 1996). To maintain this short Mn–Mn distance, the core contains two μ -oxo (O^{2-}) bridges. For catalase, the Mn(II)–Mn(II) separations are much larger, separated by ~3.7 Å for the aquo bridged form (Dismukes 1996). The water labeled O1 in the 5S rRNA (Fig. 4B) is a likely candidate for deprotonation, as it is located just 1.8 and 1.9 Å from Mg₁ and Mg₂, respectively. This distance is 0.3 Å shorter than a typical Mg–H₂O bond and 0.4 Å shorter than the next shortest bridging ligand. The other two bridges have 2.2 Å and 2.4 Å contacts to the Mg ions and might reflect hydroxo and aquo bridges, respectively. Insufficient small molecule model chemistry is currently available for Mg(II) to predict the actual protonation state of this core based on the observed distances.

The coordination chemistry of the dimagnesium center in the 5S rRNA is interesting. In the other Mg(II) sites described above, the coordination spheres of the Mg ions are believed to be quite close to octahedral. This generality holds true for the mononuclear Mg sites in the high-resolution 5S rRNA fragment as well. However, deformation of the octahedral geometry is much more evident in this dinuclear center. The coordination sphere of Mg₁ is only slightly distorted such that one of the bridging waters (O2) is pulled out of the equatorial plane consisting of O1, O2, O4, and O5. The bridging water located *trans* to the axial phosphate is significantly elongated relative to the other bridging ligands and may reflect a *trans* influence (Huheey 1983) in the chemistry of Mg(II). The geometry around Mg₂ is much more distorted. If one defines the equatorial plane as containing O1, O2, O6, and O7, the O_p–Mg₂–O3 bond angle is only 160° instead of the ideal 180°. The elongation of the monatomic bridge *trans* to the phosphate oxygen is also observed at this metal center. Both sites of phosphate coordination occur in the optimal, anti, out-of-plane geometry. The source of the distortion is likely to be the necessity to satisfy the coordination requirements of both Mg ions simultaneously.

By looking at these sites, it is clear that we are beginning to see patterns in the coordination of metal ions to specific binding sites based on X-ray crystallographic studies. A variety of metal ions will often be able to occupy the same site. The manner in which they do so, however, depends on the properties of the particular ion. From this information, it is clear that metal substitution chemistry will allow the identification of general sites quite well, but exact information about the way Mg(II) coordinates to the RNA will not be obtained through these studies. As structural data become available on larger and more complicated RNAs, more variation in the types of sites will likely be observed. In all RNAs, the majority of the sites appear to be mononuclear, but higher nuclearity sites do exist. High-resolution data unfortunately are going to be required for the greatest understanding of these interactions so that the coordinated water molecules and the geometry around the metal center can be observed.

SPECTROSCOPIC METHODS TO STUDY METAL-ION-BINDING SITES IN RNA

NMR has great potential for the localization of metal-binding sites in relatively small RNAs. Depending on the metal ion involved, the binding event can be observed by changes in the chemical shift or through the line broadening of RNA resonances. This methodology unfortunately requires the assignment of resonances. Currently, only relatively few RNAs have been studied at this level of detail, so limited data on metal binding are available.

The more common NMR approach is the use of paramagnetic metals such as Mn(II) (Bertini and Luchinat 1986). When these ions bind to the RNA, nearby atoms experience enhanced relaxation and the line broadening with a distance dependence of r^{-6} from the metal-binding site. The potential to locate binding sites using this technique is pretty clear, but the lack of data at very short distances from tightly bound ions often makes it difficult to determine the exact binding mode within the site. This technique has been applied successfully to metal binding adjacent to GU and GG sites embedded within helices (Ott et al. 1993; Allain and Varani 1995). In these studies, Mn(II) was found to be in fast exchange between its free and bound states, since substoichiometric concentrations of Mn(II) affected the entire RNA signal.

A second NMR approach relies on the chemical shifts of RNA resonances that change upon metal binding. This technique has an advantage over paramagnetic relaxation experiments in that it allows Mg(II) itself to be used. Unfortunately, the results can be difficult to interpret. Upon addition of Mg(II) to a sample, many signals may change. The main problem

is determining whether the altered spectra result from a specific binding event or a global conformational change. An example of the successful application of ^{31}P NMR in this manner involves a study of the hammerhead ribozyme where one of the Mg(II)-binding sites was localized by this method and the binding constant for the interaction determined (J.P. Simore and A. Pardi, unpubl.).

NMR has also been used to further probe the solution conformation of a metal-binding site that was observed in the P4-P6 crystal structure. In this example, the exchange inert compound $[\text{Co}(\text{NH}_3)_6]^{+++}$ was used to mimic a hydrated Mg ion (Kieft and Tinoco 1997). Observation of five NOE contacts between the ammine protons and the RNA allowed the modeling of the exact position of the metal ion in the major groove of the RNA fragment. In this case, the position and orientation of the cobalt within the binding site is much better determined than in the X-ray structure where the inner-sphere ligands were not located. The $[\text{Co}(\text{NH}_3)_6]^{+++}$ was found to be in fast exchange (on the NMR time scale) between the bound and unbound states, even though the interaction was specific and reasonably tight. Other NMR experiments that have not been fully utilized in RNA biochemistry employ metal ions that are NMR active. These metals include species like ^{113}Cd . The Cd(II) substitution has been used very successfully in the study of Zn(II)-binding proteins since NMR can provide information regarding the ligands that coordinate to the metal ion (Summers 1988).

Many other biophysical techniques can be used to extract important information regarding an established metal-binding site. One set of data that is currently of great interest is the specificity of a site as derived from the relative binding affinities in different ions. The easiest way to extract these data is to perform competition experiments. Luminescence spectroscopy recently has been used to probe the specificity of the G5 metal-binding site of the hammerhead ribozyme (Fig. 2B) (A.L. Feig et al., in prep.). Since Tb(III) binds specifically to this site and has a sensitized luminescence spectrum while bound, the extent of binding can be probed as a function of the concentration of a competitor. It is still unclear exactly what property of the ion the RNA recognizes, but it clearly does so with great specificity, as there is a 10^3 difference in the binding affinity at this site for the six different divalent ions tested.

The biophysical techniques that allow spatial localization are obviously important for the study of metal ions in RNA systems. The current focus in the field has been on finding the metal-binding sites. Once a sufficient number of examples are available, the next phase of these projects is going to turn to the more detailed probing of the coordination sphere

and role of these ions. A host of spectroscopic techniques have been developed for the study of metal ions (Drago 1977). These techniques have been applied quite successfully to the protein systems, but with much more limited usage within the area of nucleic acid biochemistry (Lippard and Berg 1995). Some of these methods have a relatively narrow range of metal ions that are suitable for study. As we learn more about the specificity of RNA-binding sites, it is hoped that we will come to a point where we will be able to either predict the metal-binding sites that will be affected under a certain set of conditions, or to control the population of individual sites.

BIOCHEMICAL APPROACHES TO LOCATE METAL-ION-BINDING SITES

Many RNA biochemical experiments involve varying the concentration and identity of metal ions. Most, however, involve examining some bulk property of the RNA and only indirectly conclude information about the location of the metal ions. Very few methods actually attempt to locate the binding site.

One of the first methods used involves ion-specific cleavage of RNA. This method relies on the fact that when metal ions such as Pb(II) or Eu(III) with sufficiently low pK_a values bind to RNA, they sometimes induce cleavage by deprotonating a nearby 2'-OH that subsequently attacks the adjacent phosphodiester bond. This method is best exemplified by cleavage at position 17 of tRNA^{Phe} by metal ions bound at the site shown in Figure 1B. Clearly, this cleavage is dependent on the coordination of the metal ion, and the site of cleavage can be slightly modulated by changing the metal ion used in the experiment (Ciesiolka et al. 1989; Michalowski et al. 1996a). Cleavage sites on other RNAs have been mapped, purportedly identifying the location of metal-ion-binding sites (Zito et al. 1993; Winter et al. 1997). However, this methodology is not ideal because (1) the site of cleavage may not be near the metal-ion-binding site in the primary sequence, and (2) not all metal-binding sites lead to cleavage. This method is better suited to assay for structural changes upon mutagenesis similar to other chemical modification reagents (Ciesiolka et al. 1989; Behlen et al. 1990; Michalowski et al. 1996a,b).

Currently, the most effective biochemical approach to locate metal-ion-binding sites in RNA involves the selective replacement of oxygen atoms suspected of being ligands to metal ions by sulfur atoms. The development of powerful chemical (Eckstein 1991) and enzymatic (Ruffner and Uhlenbeck 1990) synthesis methods has made this approach feasible. The most common substitution used for these experiments is the

R_P phosphorothioate, which can be prepared by *in vitro* transcription. Both the R_P and the S_P isomers are prepared by chemical synthesis and can often be separated by HPLC methods if the oligos are sufficiently short (Slim and Gait 1991). Other oxygen atoms that have been specifically substituted with sulfur include the 3'-bridging (Sun et al. 1997; Weinstein et al. 1997) and 5'-bridging (Piccirilli et al. 1993; Kuimelis and McLaughlin 1995, 1996; Zhou et al. 1996, 1997) phosphate oxygens, the keto oxygens of U and C (^{45}U , ^{45}C , ^{25}C) (Wang and Ruffner 1997), and the 2'-OH group (Hamm and Piccirilli 1997). Varying degrees of efficacy have been observed with respect to the modulation of the catalytic activity and metal ion specificity with these modifications.

The basic chemistry of the experiment derives from hard and soft acid base theory. The idea is that the sulfur modification will reduce the affinity of a given oxygen ligand for a Mg(II) ion. Since Mg(II) is an extremely hard Lewis acid ($\eta = 47.59$), binding to the sulfur atom will be disfavored. If this ligand is critical for positioning a metal ion involved in the folding or catalysis of the ribozyme, activity will be lost. In experiments often referred to as rescue experiments, the addition of softer metals, such as Mn(II) ($\eta = 9.02$) or Cd(II) ($\eta = 10.29$), can restore activity by interacting with the sulfur atom and thus replace the Mg ion. The choice of a rescue metal should be governed by the hardness parameter η listed in Table 2, but several ions should be tested because the structural details of the binding site and a variety of experimental parameters will dictate whether any given ion will be able to successfully replace Mg(II) in a particular site. In early experiments, the rescue metal was used to the exclusion of other metal ions in solution (Dahm and Uhlenbeck 1991). More recent work has shown that better data result from the use of lower concentrations of the rescue metal in the presence of a significant excess of Mg(II) to assure that the other metal-ion-binding sites contain Mg(II) and are therefore not perturbed by the presence of the softer ion (Christian and Yarus 1993; Piccirilli et al. 1993; Peracchi et al. 1997). In model chemistry that used adenosine nucleotide di- and triphosphates and their thiophosphate analogs, where the individual binding constants for a variety of ions could be measured accurately, Mg(II) showed a 31,000-fold preference for binding the oxygen rather than the sulfur (Pecoraro et al. 1984). In the same experiments, Cd(II) exhibited a 55-fold preference for the sulfur and Mn(II) showed approximately equal affinity for oxygen and sulfur ligands. Qualitatively similar findings were found for a series of metal ions with respect to their affinity for AMP^{2-} and AMPS^{2-} (Sigel et al. 1997).

Phosphorothioate rescue experiments have been used in a number of ribozyme systems, including the hammerhead (Dahm and Uhlenbeck

1991; Slim and Gait 1991; Peracchi et al. 1997), the hepatitis δ virus (Jeoung et al. 1994; Fauzi et al. 1997), hairpin (Chowrira and Burke 1991, 1992), group I intron (Waring 1989; Christian and Yarus 1993; Piccirilli et al. 1993), group II intron (Chanfreau and Jacquier 1994; Podar et al. 1995), and RNase P (Harris and Pace 1995; Warnecke et al. 1996). These experiments provide the most useful data when activity in Mg(II) alone is reduced but the addition of Mn(II) or Cd(II) restores catalysis. This combination of results is interpreted as strong evidence for a metal-binding site that utilizes the specific phosphate oxygen as an inner-sphere ligand. When one phosphorothioate isomer but not the other displays this behavior, the conclusions are even more strongly supported, as is the case with the cleavage site phosphorothioate substitution in the hammerhead ribozyme (Koizumi and Ohtsuka 1991; Slim and Gait 1991) because it controls for the structural deformations that might have occurred as a result of this substitution. In certain cases, activity is lost upon incorporation of the phosphorothioate into the ribozyme at a certain position, but addition of a rescue metal does not restore activity (Strobel and Shetty 1997). When this behavior is observed, a role for the phosphate oxygen in metal binding is not excluded, but it must be remembered that even though the change is a single oxygen atom to a sulfur, significant perturbation of local structure can result. The charge localization on a phosphorothioate residue is also different from a standard phosphodiester, residing primarily on the sulfur as opposed to being distributed evenly between the nonbridging oxygen atoms (Frey and Sammons 1985). Furthermore, the phosphorus–sulfur bond is longer than a typical phosphate–oxygen interaction. Together, these structural and electronic differences can lead to misalignment of functional groups required for catalytic activity irrespective of metal binding. In a few cases, data from phosphorothioate rescue experiments can be related to metal binding at the site observed crystallographically. One example of such corroboration comes from the p9 site in the hammerhead ribozyme (Fig. 2C) (Peracchi et al. 1997). Another example comes from the P4-P6 structure (Cate and Doudna 1996; Cate et al. 1997). Rescue experiments therefore appear to accurately predict metal-binding sites that contain phosphate–oxygen ligation.

There are a few caveats and potential biases in sulfur modification experiments. The main limitation with the use of phosphorothioate chemistry is that only metal ions that contain inner-sphere contacts to the phosphate backbone are sensitive to this class of experiments. This technique may find many of the important metal-binding sites, but it can never find all of them because some of the site(s) of interest may not contain these ligands. A further potential problem is referred to as “recruitment.” As the

name implies, there is the possibility that by making the modification to the RNA, a metal-binding site was created that did not exist in the parent molecule. Recruitment cannot formally be excluded as a possible side effect of these modifications; however, the incorporation of phosphorothioates in most positions in ribozymes does not affect the catalytic rate even when thiophilic metal ions are added. Thus, recruitment is not a particularly serious problem. Systematic probing of phosphorothioates in RNAs that are easily characterized will be required to determine the significance of this potential artifact. Small-molecule modeling studies can also be used to address this problem.

The use of competitive inhibition experiments is another versatile method to identify metal-binding events that are critical for activity in a catalytic ribozyme system. This technique answers questions about metal specificity in a critical site, but alone does not allow the identification or localization of the metal-binding site(s) involved in the inhibition event. The choice of inhibitors should therefore be made with the problem of localization in mind. Luminescent metal ions are generally relatively easy to localize based on energy-transfer experiments, the utility of which was originally demonstrated on tRNAs (Kayne and Cohn 1974; Wolfson and Kearns 1974; Draper 1985). In principle, localization of the sites of Cr(II) or Co(II) binding to RNA can also be accomplished by oxidizing them to Cr(III) or Co(III) in situ because the ligand exchange rates of the +3 ions are sufficiently slow that the complex remains intact during analysis (Danchin 1973, 1975).

METAL IONS IN THE RNA WORLD

By combining our limited knowledge of metal-ion-binding to contemporary RNAs and our more extensive knowledge of metal-ion-binding to proteins, it is possible to speculate on the role of metal ions in prebiotic molecular evolution. It seems clear that specifically bound metal ions co-evolved with RNA molecules. Many of the mononuclear sites in Table 5 are formed with, or can be engineered into, small RNA fragments. Since such sites are highly hydrated and contain limited direct contact with the RNA, the observed affinities are only moderate, in the 1–1000 μM range. These sites are also expected to show limited specificity, predominantly dictated by the chemical nature of the ligands. Furthermore, in these examples, the RNA structures themselves are likely to be quite flexible and can accommodate a variety of metal ions with only minor distortions to the overall RNA fold. These minimalist sites are sufficient to stabilize the secondary and tertiary structures observed in these motifs.

The metal ion sites generated on small RNAs appear to be capable of facilitating a variety of different types of chemistry. Activities range from the transesterification and hydrolytic reactions of small ribozymes (Pyle 1996; Sigurdsson et al. 1998) to the more exotic porphyrin metalation (Conn et al. 1996) and Diels-Alder condensation reactions (Tarasow et al. 1997) catalyzed by aptamers produced from *in vitro* selection experiments. These small RNAs have only limited amounts of structure and therefore are likely to position the catalytic metal ions by only a few points of contact. The relatively modest rate enhancements supported by catalytic RNAs such as these probably reflect the types of species that first evolved from random polymerization events. Very active metal ions might have assisted in this process but would have increased the danger of side reactions that would accidentally damage the catalyst.

A striking difference between most RNA metal-binding sites studied thus far and those seen in proteins is the degree of hydration. Both structural and catalytic metal-ion-binding sites in proteins are predominantly dehydrated (Lippard and Berg 1995). Water molecules occasionally appear in the coordination spheres of these metal ions, but in these cases, they are often believed either to be displaced by the substrate when it enters the active site or to take part in the catalytic mechanism of the enzyme. Such protein sites also bind their metal ions much more tightly than the RNA systems. In fact, tight binding is a requirement for dehydrated sites, since there is a characteristic energy (ΔH_{hyd}) associated with the hydration of any ion. The net binding energy upon coordination of the ion must account for the energetic cost of dehydration. The question arises, Why are such dehydrated sites not observed in RNAs?

One possibility is that metal-binding sites in RNAs are intrinsically different from those in proteins. RNA has a much more limited set of ligands to use in generating a specific metal-binding pocket. Amino acid side chains containing thiols and thioethers are well suited to binding a variety of softer metals. In addition, the carboxylate side chains provide anionic ligands with great versatility in their potential modes of coordination. They can act as either terminal or bridging ligands and bind in either monodentate or bidentate geometries. The nucleotides, on the other hand, are much larger and more rigid than the corresponding amino acids. The anionic ligand in RNA, the nonbridging phosphate oxygen, is an integral component of the backbone and therefore is more limited in its conformational freedom than the aspartate and glutamate carboxylate groups. The heterocyclic ring nitrogens and the keto oxygens from the bases are held in rigidly planar orientations by the aromatic rings. This geometric constraint severely limits the ability of an RNA to compactly

encompass a metal ion and provide more than facial coordination and therefore complete dehydration. It also explains why the most specific metal-binding sites are not in the Watson-Crick base-paired regions of the structure where the conformation is too constrained. Instead, metal-ion-binding sites are clustered in regions of extensive distortion from the A-form RNA helices.

There is also the question of the folding of RNAs relative to that of proteins. It is possible that in RNAs there is insufficient energy in the folding and metal-binding process to completely displace the waters of hydration around a metal ion. It has been suggested that in contemporary RNAs, modified nucleotides might be present to assist in metal ion binding (Agris 1996). A more straightforward possibility, however, is that most RNAs studied to date are structurally too simple. In these RNAs, most residues involved in metal ion binding are solvent-exposed. Thus, the RNAs have no real inside comparable to the hydrophobic core of a protein. The largest RNA crystallographically characterized to date is the P4-P6 domain. On the basis of that structure, it was proposed that an ionic core may substitute in RNA folding for the hydrophobic core of proteins such that the 3° structure assembles around a fixed number of discrete metal-binding sites (Cate et al. 1997). Even in this structure, however, the most buried of the metal-binding sites are significantly hydrated.

It could be that all metal-ion-binding sites in RNA are at least partially hydrated. One can imagine several advantages to using hydrated ions within the ionic core of a large RNA. Hydrated ions would span larger voids than dehydrated ions and allow looser packing of secondary structure elements. The hydrated ion also can accommodate a wide range of structural interactions through its orientation of the water molecules as compared to direct coordination of metal ions at every site. In addition, the energy associated with deforming the outer-sphere interactions should be significantly less than what would be observed for distorting the inner-sphere coordination. A consequence of RNAs having a core of hydrated ions is that one might expect this core to be much more dynamic than the hydrophobic core of a protein.

In the modern protein world, metal cofactors are associated with a variety of reaction types, including electron transfer, redox chemistry, and hydrolysis reactions. Transesterification and hydrolytic activities, however, are the primary catalytic behaviors observed in ribozymes. Did these other catalytic activities not develop until the dawn of the protein world, or are there undiscovered natural catalytic RNAs that are the ancestors of the early redox enzymes? Through the use of *in vitro* selection experiments, the scope of RNA catalysis has been significantly broadened. RNA

is almost certainly capable of catalyzing these other classes of reactions, but it is still unclear whether there are naturally occurring examples. Such an enzyme would likely use a metal ion cofactor other than Mg(II), so the search for RNA molecules that naturally use alternative ions is of significant interest. A recent selection experiment showed that a single base change results in an altered metal ion specificity for RNase P (Frank and Pace 1997). It is clear from this result that catalytic RNAs retain the ability to adapt to an everchanging environment, using the resources available to evolve and to overcome evolutionary pressures. Were RNAs to have evolved out of an environment devoid of metal ions, they probably would have found a way around the problems of folding and generating reactive functional groups. The primordial soup and all cellular environments that have evolved subsequently contained a variety of ions, however. Given the availability of metal ions, they will certainly play a significant role in the biology of current and future RNAs.

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