Cooperativity in Low-affinity Mg\textsuperscript{2+} Binding to tRNA*

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The Pb\textsuperscript{2+}-catalyzed cleavage of tRNA\textsuperscript{Phe} has been used to probe the effect of Na\textsuperscript{+} and Mg\textsuperscript{2+} binding to tRNA. Na\textsuperscript{+} is a noncompetitive inhibitor of the Pb\textsuperscript{2+}-catalyzed cleavage. Millimolar Mg\textsuperscript{2+} is also a noncompetitive inhibitor. Analysis of the Mg\textsuperscript{2+} data show that at least two sites are involved in binding and that there is an interaction between the sites (cooperativity). Low-affinity Mg\textsuperscript{2+} binding is thus different from "weak" and "strong" Mg\textsuperscript{2+} binding to tRNA characterized previously. We postulate that the alterations induced by low-affinity Mg\textsuperscript{2+} binding in tRNA mimic to some extent those brought about in RNA by the interaction with a protein factor and that at appropriate [Mg\textsuperscript{2+}] the whole structure of tRNA is able to respond in a concerted way to a signal from the environment such as aminoaacetylation or codon binding.

Interest in the effect and role of Mg\textsuperscript{2+} in promoting RNA function has been rekindled recently. 5–10 mM Mg\textsuperscript{2+} is required for the autoexcision and autocyclization of the RNA intervening sequence of Tetratrichymena (1); at greater than 20 mM Mg\textsuperscript{2+}, the RNA moiety of RNase P performs the catalytic function of the enzyme in the absence of its protein subunit (2); 16 S and 23 S rRNAs form a specific bimolecular complex at a higher [Mg\textsuperscript{2+}] than that required for 30 S and 50 S subunit association (3). Work in the tRNA field is equally rich in examples. Higher [Mg\textsuperscript{2+}] may compensate for the absence of protein factors in binding of tRNA to ribosomes (4). Alterations of tRNA tertiary structure by codon binding (5) or by tRNA aminoaacetylation (6) are also promoted by millimolar Mg\textsuperscript{2+}. A stimulatory effect of Mg\textsuperscript{2+} is observed in the tRNA binding to template-free ribosomes (7) as well as in the codon-induced tRNA dimerization in the absence of ribosomes (8, 9). These observations imply the existence of low-affinity Mg\textsuperscript{2+} binding (i.e. occurring in the millimolar range) which is important for the functional competence of RNA molecules. It is therefore relevant to characterize this Mg\textsuperscript{2+} binding, since it may bring light to bear on the active structure of RNA and on the specific protein:RNA interactions involved in RNA function.

Generally, two approaches are used in studies of ligand binding to macromolecules. These involve either the measurements of free and free plus bound ligand concentrations or the titration of a parameter of the system which is sensitive to ligand binding. The first approach is reliable as long as the effect of bound ligand can be determined in the presence of a high free-ligand concentration. Generally, weak binding sites in the presence of multiple, stronger sites cannot be assayed. The second approach is useful only if the ligand induces observable changes in the parameter to be measured. In the case of tRNA and model polynucleotides, however, the parameters (such as absorption, ellipticity, or fluorescence) which are sensitive to RNA folding are usually saturated at millimolar Mg\textsuperscript{2+} (10–13).

In the present study, we analyze Na\textsuperscript{+} and Mg\textsuperscript{2+} binding to yeast tRNA\textsuperscript{Phe} by the Pb\textsuperscript{2+} mediated cleavage reaction. Pb\textsuperscript{2+} is known to catalyze hydrolysis of tRNA (14) or 5 S RNA\textsuperscript{1} at specific sites. Recently, a crystallographic study characterized the binding site for Pb\textsuperscript{2+} in yeast tRNA\textsuperscript{Phe} (15). This binding leads to the cleavage of the phosphodiester bond between dhU\textsubscript{17} and G\textsubscript{18}, as previously found in solution (14). We observe that at increasing [Na\textsuperscript{+}] and [Mg\textsuperscript{2+}], cleavage is inhibited. Analysis of the inhibition of this "metalloenzymatic" reaction fortuitously permits the characterization of the low-affinity Mg\textsuperscript{2+} binding to tRNA since inhibition requires relatively high [Mg\textsuperscript{2+}].

MATERIALS AND METHODS

Yeast tRNA\textsuperscript{Phe} was obtained from Boehringer Mannheim. In vitro labeling with \textsuperscript{32}P and purification were performed as previously described (16). tRNA was located on the gel by autoradiography and eluted using a 0.3 M NaCl, 0.1% sodium dodecyl sulfate solution. NH\textsubscript{4}\textsuperscript{+} ions must be avoided since they seem to poison the Pb\textsuperscript{2+} cleavage. Such contamination in tRNA samples may be tested by measuring the effect of tRNA concentration on the extent of cleavage. Reactions have been carried out at 0 °C in 56 mM Hepes,\textsuperscript{2} pH 7.4, 0.5 mM MgCl\textsubscript{2}, and variable amounts of NaCl or in the same buffer containing 70 mM Na\textsuperscript{+} when MgCl\textsubscript{2} concentration was varied. Under these conditions, tRNA tertiary interactions are maintained (17). tRNA\textsuperscript{Phe} concentration was 1 pg/\muL (adjusted with unlabeled tRNA). The reaction was started by the addition of 0.2 × final volume of 5-fold concentrated solution of Pb acetate (Anachema Chemicals Ltd., Toronto, Canada) prepared just prior to the experiment in freshly distilled water. The reaction was stopped by the addition of an excess of EDTA, and the sample was vacuum-dried, dissolved in deionized distilled water, and electrophoresed on a 3% polyacrylamide gel. The gel was stained with 0.5% ethidium bromide and destained with water.

RESULTS

The incubation of tRNA\textsuperscript{Phe} with Pb\textsuperscript{2+} leads to a single cleavage of tRNA at position 17 (Fig. 1, left), as had been previously shown in crystallographic (15) and solution (14) studies. However, prolonged incubation in excess of Pb\textsuperscript{2+}

1 D. Labuda, K. Nicoghosian, and R. Cedergren, unpublished observations.

2 dhU represents dihydrouridine. Subscripts (dhU\textsubscript{17}, G\textsubscript{18}, etc.) denote the nucleoside position in the primary structure of tRNA\textsuperscript{Phe}. P\textsubscript{19} represents the phosphate residue of nucleotide 19.

3 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
eventually leads to the complete hydrolysis of the polyribonucleotide chain (18). Similarly, secondary cuts at positions 15 and 16 manifest themselves after an overnight incubation in 1 mM Pb²⁺ (data not shown). As in Ref. 14, we have not observed hydrolysis at position 22 which is seen in crystalline tRNA₃Ihe (15). In the experiments reported below, the extent of Pb²⁺ cleavage has been measured with ³²P-labeled tRNA₃phe using small polyacrylamide gels (Fig. 1, right), after reaction times of 25 or 30 min (see Fig. 2).

The logarithmic plot of cleavage versus time shows that the reaction follows pseudo-first-order kinetics (Fig. 2). According to the proposed mechanism (15), Pb²⁺ binds to the specific site in tRNA and acts as a metalloenzyme, hydrolizing the phosphodiester linkage. The overall scheme for this reaction may thus be written as follows.

$$\text{tRNA} + \text{Pb}^{2+} \xrightarrow{k_{1a}} \text{tRNAPh} \xrightarrow{k_{3}} \text{RNA}_{\text{frag}} + \text{Pb}^{2+}$$

(1)

The rate of cleavage is

$$v = k_{1a}[\text{tRNAPh}] = k_{3} \frac{K_{Rs}[\text{Pb}^{2+}]}{K_{Rs}[\text{Pb}^{2+}] + 1} - T_t = k_{oa}T_t$$

(2)

where $T_t$ is the concentration of intact tRNA at a given time $t$ and $K_{Rn} = k_1/k_3$ is the binding constant of Pb²⁺ in its cleavage site (we may assume $k_2 \gg k_3$; see Table I). The observed rate constant $k_{oa}$ can be obtained from the following relation (see Fig. 2).

$$\log \frac{T_t}{T_{t=0}} \left( \frac{\text{cpm}_{\text{int}} - \text{cpm}_{\text{frag}}}{\text{cpm}_{\text{int}}} \right) = -k_{oa}t/2.303$$

(3)

The reciprocal of $k_{oa}$ is dependent upon the presence of a competitive or a noncompetitive inhibitor $I$ as described by Equations 4 and 5, respectively:

$$k_{oa}^{-1} = \frac{1}{k_{3}} \frac{K_{Rs}[\text{Pb}^{2+}]}{K_{Rs}[\text{Pb}^{2+}]} + 1 \frac{1}{k_{3}} \frac{K_{Rs}[\text{Pb}^{2+}]}{K_{Rs}[\text{Pb}^{2+}]} K_{I}$$

(4)

$$k_{oa}^{-1} = \frac{1}{k_{3}} \frac{K_{Rs}[\text{Pb}^{2+}]}{K_{Rs}[\text{Pb}^{2+}]} + 1 \frac{1}{k_{3}} \frac{K_{Rs}[\text{Pb}^{2+}]}{K_{Rs}[\text{Pb}^{2+}]} K_{I}$$

(5)

where $K_{I}$ (in M⁻¹) denotes the binding constant of $I$.

In the case of Na⁺ inhibition, the values of $k_{oa}^{-1}$ obtained at three different [Pb²⁺] show a linear dependence on [Na⁺] (Fig. 3A). Both the slopes and the abscissa intercepts (compare Equations 4 and 5) indicate that sodium ions inhibit the cleavage reaction in a noncompetitive way. The effect of Na⁺ is then described by a simple, single-site noncompetitive inhibition model.

In the case of the Mg²⁺ data shown in Fig. 4A, the $k_{oa}$ shows a positive curvature when plotted as a function of [Mg²⁺]. This implies that more than one Mg²⁺ is involved in the inhibition of cleavage. Generally, a multisite inhibition can be analyzed using the Hill approximation (e.g. Chapter VIII in Ref. 19). In such a case, the product $K_{I}^{n}$ in Equation 4 or 5 becomes $K_{I}^{n}$, where $n$ is the Hill coefficient and $K_{I}$ is the apparent inhibitory binding constant. We define $k_{I}$ as $k_{oa}$ measured in the absence of inhibitor, i.e. $k_{I} = k_{oa}K_{Rs}[\text{Pb}^{2+}]/(K_{Rs}[\text{Pb}^{2+}] + 1)$. By rearranging the modified Equation 5 (noncompetitive inhibition), we obtain the expression which in its logarithmic form permits the derivation of $n$ and $K_{I}$:

$$-\log \frac{k_{I} - k_{oa}}{k_{oa}} = -n \log [I] - \log K_{I}$$

(6)

Data plotted according to this equation are shown in Figs. 3B and 4B.

As can be seen in Fig. 4B, the Mg²⁺ data points obtained at different [Pb²⁺] fall on the same line as with the Na⁺ data in
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### TABLE I
Parameters of the inhibitory Na\(^+\) and Mg\(^{2+}\) binding to yeast tRNA\(^{pk}\) and the specific cleavage by Pb\(^{2+}\) (in 50 mM Hepes, pH 7.4, at 0°C) derived from the titration data shown in Figs. 3 and 4 and analyzed as described in the text.

<table>
<thead>
<tr>
<th>Inhibitor (titrant)</th>
<th>[Na(^+)]</th>
<th>[Mg(^{2+})]</th>
<th>(k_i)</th>
<th>(K_{pp})</th>
<th>(K_{nc})</th>
<th>(2K_{II})</th>
<th>(%K_{II})</th>
<th>(K_i)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>0.0005</td>
<td>15.4</td>
<td>1750</td>
<td>20.5</td>
<td>20</td>
<td>&lt;1</td>
<td>19.7</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>0.07</td>
<td>37.5</td>
<td>1950</td>
<td>10.2(^{2+})</td>
<td>116(^{2+})</td>
<td>40.7(^{2+})</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Because of cooperativity in Mg\(^{2+}\) binding, the values of \(2K_{1i}Mg\) and \(\%K_{2i}Mg\) should be taken as a measure of affinity for the first and second Mg\(^{2+}\), respectively.

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Fig. 3. Analysis of the Pb\(^{2+}\) cleavage inhibition by Na\(^+\) ions at 0.25 (O), 0.5 (C), and 0.75 mM (O) Pb acetate. A, dependence of \(k_\text{ob}\) upon [Na\(^+\)] in 50 mM Hepes pH 7.4, 0.5 mM MgCl\(_2\). The same data are plotted according to Equation 6 in B and according to Equation 7 in C using \(k_L = 0.0199, 0.0303,\) and 0.0389 min\(^{-1}\) for 0.25, 0.5, and 0.75 mM Pb acetate data, respectively. The parameters compiled in Table I were obtained by linear regression of the experimental points (solid lines). For clarity, the error bars are omitted. The error in \(k_\text{ob}\) determination is estimated to be ±15%.

Fig. 4. Analysis of the Pb\(^{2+}\) cleavage by Mg\(^{2+}\) ions in the presence of 0.25 (O), 0.5 (C), and 0.75 mM (O) Pb acetate. A, dependence of \(k_\text{ob}^{-1}\) upon [Mg\(^{2+}\)] in 50 mM Hepes, pH 7.4, containing 70 mM Na\(^+\). The data points represent an average of two to three determinations, with estimated error of ±15%. B and C, as in Fig. 3, using \(k_L = 0.0087, 0.0132,\) and 0.0157 min\(^{-1}\) for 0.25, 0.5, and 0.75 mM Pb acetate data, respectively. The parameters compiled in Table I were obtained by linear regression (solid lines) from the data in B and C and were used to calculate the curves (solid lines) in A. Dotted and dashed lines demonstrate the behavior assuming either independent binding or full cooperativity and were calculated using \(K_{II} = K_{I} = 16 \text{ M}^{-1}\) and \(K_{II} = K_{I} = 160 \text{ M}^{-1}\), respectively.
Mg"⁺ can be then described by a two-site noncompetitive inhibition scheme.

Values of parameters obtained from the analysis of Na⁺ and Mg"⁺ inhibition according to the models presented above are compiled in Table I. K_{Na} of 150 M⁻¹ is also obtained from independent measurements at different [Ph⁺] (see Fig. 2). That the affinity for the second Mg"⁺ is greater than for the first (K_{Mg Na} < K_{Mg Mg}) and/or that the Hill coefficient is equal to 1.7 demonstrates the Mg"⁺ binding to be cooperative. It was thus interesting to compare the experimental data with the behavior of Mg"⁺ binding in the case of "fully" cooperative (K_{Hill} < K_{Hill}) or the independent two site binding model (K_{Hill} = K_{Hill}). The simulated curves are included in Fig. 4. Given the fact that the calculated curves according to an independent binding model (dotted lines) only slightly deviate from linearity in the plot of Fig. 4A and that the Hill coefficient derived from the plot in Fig. 4B is very close to 1, it would be difficult with real data to differentiate this model from a single-site inhibitory binding (cf. Fig. 5). The same argument applies for the plot in Fig. 4C if one assume K_{Hill} > K_{Hill}. This means that the Na⁺ data are also consistent with a multisite inhibitory binding scheme provided that K_{Na Na} > K_{Na Na}, even though a single-site inhibition is sufficient for their description. The K_{Na Na} and K_{Na Na} values consistent with a two-site scheme are included in Table I for comparison with the parameters of Mg"⁺ binding.

In the above analysis, we assume that the inhibition is due to the binding of the first Mg"⁺. An alternative model could be entertained assuming that the inhibition takes place only after the second Mg"⁺ is bound. Consideration of results with Na⁺ data are also consistent with a multisite inhibitory binding with the affinity constants lower than those reported for the weak and strong binding. Based on these characteristics we call it low-affinity Mg"⁺ binding. As shown in Ref. 27, the independent character of weak sites does not preclude a negative curvature in Scatchard plots due to the polyelectrolyte nature of RNA. In other words, the apparent binding constant derived from the binding curve measured at high level of saturation with ligand cations will be lower than the average value obtained from the entire titration curve including the electrostatic effect. High [Mg"⁺] required for the inhibition of cleavage implies that most Mg"⁺-binding sites in tRNA are already occupied, which may explain the low values of K_{Mg Mg} observed. Accordingly, due to electrostatic repulsion, the apparent binding constant should decrease with each additional binding of ligand cations to tRNA. Such is the case with Na⁺ inhibitory binding constants (K_{Hill} > K_{Hill}, Table 1), which can be compared with the values obtained by fluorescence titration where decreasing apparent K_{Na Na} values (27, 24, and <1 M⁻¹) were observed (28).

Experiments reported here reveal the existence of a cooperative Mg"⁺ binding with the affinity constants lower than those reported for the weak and strong binding. Based on these characteristics we call it low-affinity Mg"⁺ binding. As shown in Ref. 27, the independent character of weak sites does not preclude a negative curvature in Scatchard plots due to the polyelectrolyte nature of RNA. In other words, the apparent binding constant derived from the binding curve measured at high level of saturation with ligand cations will be lower than the average value obtained from the entire titration curve including the electrostatic effect. High [Mg"⁺] required for the inhibition of cleavage implies that most Mg"⁺-binding sites in tRNA are already occupied, which may explain the low values of K_{Mg Mg} observed. Accordingly, due to electrostatic repulsion, the apparent binding constant should decrease with each additional binding of ligand cations to tRNA. Such is the case with Na⁺ inhibitory binding constants (K_{Hill} > K_{Hill}, Table 1), which can be compared with the values obtained by fluorescence titration where decreasing apparent K_{Na Na} values (27, 24, and <1 M⁻¹) were observed (28).

The Role and Nature of Low-affinity Mg"⁺-binding Sites—It has been shown using laser-light scattering that no difference is detectable between aminoacyl-tRNA and uncharged tRNA at 1 mM Mg"⁺. The structural changes induced by tRNA-aminoacylation do appear, however, to result from cooperative Mg"⁺ binding in the millimolar range (6). Codon-induced alterations in tRNA tertiary structure also appear at millimolar Mg"⁺ and show characteristic sigmoidal dependence upon [Mg"⁺], indicating cooperativity (5). Similar Mg"⁺ dependence has been observed in codon-induced tRNA-di-
merization, extensively studied by relaxation measurements (8). In all these experiments, a relatively small ligand such as an aminoacyl residue or a codon triplet triggers changes in properties or structure of tRNA far away from its binding site. They also share a sigmoidal dependence upon $[\text{Mg}^{2+}]$ in the millimolar range; the $\text{Mg}^{2+}$ binding involved correspond an aminoacyl residue or a codon triplet triggers changes in signal from the environment such as aminoacylation or codon upon whole tRNA structure could respond in a concerted way to a site. They also share a sigmoidal dependence upon $[\text{Mg}^{2+}]$ in all these experiments, a relatively small ligand such as along the polynucleotide chain by inducing changes in the signal of signals along nucleic acid chains (8). According to this binding of spermine creates strong binding sites for divalent or polyvalent ligands may replace $\text{Mg}^{2+}$ in Mg-tRNA salt, at cations in tRNATy' biological functions of tRNA, indicating differences in the affinity $\text{Mg}^{2+}$ binding sites may be essential for its function. If low-affinity $\text{Mg}^{2+}$ binding mimics the influence of protein mechanism, indicating as well that the saturation of low-affinity $\text{Mg}^{2+}$ binding is due to low-affinity $\text{Mg}^{2+}$ binding. Thus, we believe decreased due to low-affinity $\text{Mg}^{2+}$ binding. Thus, we believe that the structural effect of low-affinity $\text{Mg}^{2+}$ binding is involved in the outer shell of the tRNA molecule rather than in its polynucleotide core. This is consistent with the recent interpretation (29) of dynamic light-scattering measurements (30) that the effect of millimolar $\text{Mg}^{2+}$ is primarily due to changes in size and shape of the counterion shield and layer of hydration in tRNA. Similarly, the neutron-scattering data (31) emphasize the importance of the extent and location of the solvent shell for biological function of tRNA.

The effect of $\text{Mg}^{2+}$ on tRNA folding can be mimicked by high monovalent-cation concentration (12, 22, 23). Also, the inhibition of cleavage is obtained by both $\text{Na}^+$ and $\text{Mg}^{2+}$. However, monovalent cations cannot replace $\text{Mg}^{2+}$ in the biological functions of tRNA, indicating differences in the biochemistry of Mg-tRNA and Na-tRNA salts. From the present study, these differences appear to be due to the interacting character of $\text{Mg}^{2+}$ sites which is manifested at millimolar $\text{Mg}^{2+}$. It seems plausible, however, that other oligo- or polyanivalent ligands may replace $\text{Mg}^{2+}$ in Mg-tRNA salt, at least to some extent. It has been shown, for example, that binding of spermine creates strong binding sites for divalent cations in tRNA$^{37}$ (32). tRNA complexed with the elongation factor responds to codon binding by alterations of tertiary structure at lower $[\text{Mg}^{2+}]$, and the effect shows less sigmoidal dependence on $[\text{Mg}^{2+}]$ than in the case of tRNA alone (33). If low-affinity $\text{Mg}^{2+}$ binding mimics the influence of protein ligands on the structure and properties of RNA, it explains why millimolar $\text{Mg}^{2+}$ compensates for the absence of protein factors and supports the activity of naked RNA molecules as known in many cases (1-4, 7). Analysis of RNA properties at high $[\text{Mg}^{2+}]$ could help therefore to understand the structure of RNA in a RNA-protein complex and the molecular basis of its activity.

REFERENCES