Symmetric K\textsuperscript{+} and Mg\textsuperscript{2+} Ion-binding Sites in the 5 S rRNA Loop E Inferred from Molecular Dynamics Simulations

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Potassium binding to the 5 S rRNA loop E motif has been studied by molecular dynamics at high (1.0 M) and low (0.2 M) concentration of added KCl in the presence and absence of Mg\textsuperscript{2+}. A clear pattern of seven deep groove K\textsuperscript{+} binding sites or regions, in all cases connected with guanine N7/O6 atoms belonging to GpG, GpA, and GpU steps, was identified, indicating that the LE deep groove is significantly more ionophilic than the equivalent groove of regular RNA duplexes. Among all, two symmetry-related sites (with respect to the central G·A pair) were found to accommodate K\textsuperscript{+} ions with particularly long residence times. In a preceding molecular dynamics study by Auffinger et al. in the year 2003, these two sites were described as constituting important Mg\textsuperscript{2+} binding locations. Altogether, the data suggest that these symmetric sites correspond to the loop E main ion binding regions. Indeed, they are located in the deep groove of an important ribosomal protein binding motif associated with a fragile pattern of non-Watson–Crick pairs that has certainly to be stabilized by specific Mg\textsuperscript{2+} ions in order to be efficiently recognized by the protein. Besides, the other sites accommodate monovalent ions in a more diffuse way pointing out their lesser significance for the structure and function of this motif. Ion binding to the shallow groove and backbone atoms was generally found to be of minor importance since, at the low concentration, no well defined binding site could be characterized while high K\textsuperscript{+} concentration promoted mostly unspecific potassium binding to the RNA backbone. In addition, several K\textsuperscript{+} binding sites were located in positions equivalent to water molecules from the first hydration shell of divalent ions in simulations performed with magnesium, indicating that ion binding regions are able to accommodate both mono- and divalent ionic species. Overall, the simulations provide a more precise but, at the same time, a more intricate view of the relations of this motif with its ionic surrounding.

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Keywords: X-ray crystallography; Brownian dynamics simulations; ion competition

Introduction

Although Mg\textsuperscript{2+} ions are commonly associated with folding\textsuperscript{1}, stability\textsuperscript{2}, and catalytic activity of RNA molecules,\textsuperscript{3,4} it is becoming manifest that monovalent ions (K\textsuperscript{+}, Na\textsuperscript{+},...) also play direct roles in these processes.\textsuperscript{5} For example, the ribosome requires the presence of monovalent ions \textit{in vitro}\textsuperscript{6,7} and the activity of a ribozyme is selectively increased by the presence of K\textsuperscript{+} ions.\textsuperscript{8} Furthermore, a high concentration (>1.0 M) of monovalent ions...
is known to promote a fold of tRNA molecules which probably closely resembles that determined by X-ray crystallography. More recently, it has been emphasized that monovalent ions can substantially modulate the structure of RNA molecules in the absence of Mg$^{2+}$ ions and induce the formation of important tertiary contacts. In highly refined crystallographic NMR and theoretical models, many metal ion binding sites that can specifically accommodate monovalent ions (Na$^+$, K$^+$, Rb$^+$, Cs$^+$, Ti$^+$, NH$_4^+$) have been thoroughly described. Interestingly, molecular dynamics simulations (MD) played a pioneering role in that recognition since, before the availability of precise experimental data related to the binding of monovalent ions to nucleic acids, MD studies had already led to the proposal that Na$^+$ ions can intrude into the minor groove of DNA duplexes and specifically bind to the highly electronegative anionic oxygen atoms belonging to the phosphate backbone. The dynamical behavior of these monovalent ions has already been described in detail. Here, we will concentrate on the binding of K$^+$ ions to the eubacterial LE motif in the presence and absence of Mg$^{2+}$ ions by analyzing four 11.45 ns MD trajectories. Simulation NoMg is devoid of Mg$^{2+}$ ions while simulations 4MgA and 4MgB contain each a subset of the five monovalent Mg$^{2+}$ ions. The 4MgA, 4MgB, and NoMg simulations reproduce a system containing 0.2 M of added KCl while, in order to get some clues regarding the stabilization of the LE motif induced in the absence of Mg$^{2+}$ ions by a high concentration of monovalent ions, a simulation reproducing a system containing 1.0 M of added KCl (NoMg1M) has been undertaken (Table 1).

In an earlier publication, we showed that no MD simulation protocol could reproduce the characteristics of the binuclear Mg$^{2+}$ cluster that is present in the deep groove of LE (in the crystal structure, two Mg$^{2+}$ ions are separated by an unusually short distance of 2.7 Å), and it was suggested that the detection of the bimetallic cluster by X-ray diffraction methods could be explained by a partial occupancy of each of the two Mg$^{2+}$ binding sites. A model combining a 1:2 ratio of the two 4MgA and 4MgB simulations, each excluding one of

Table 1. Characteristic simulation parameters and number of molecules present in the simulation box

<table>
<thead>
<tr>
<th></th>
<th>4MgA</th>
<th>4MgB</th>
<th>NoMg</th>
<th>NoMg1M</th>
</tr>
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<tbody>
<tr>
<td>Total length (ns)</td>
<td>11.45</td>
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<tr>
<td>Production (ns)</td>
<td>10.00</td>
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<td>Mg$^{2+}$</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cl$^{-}$ concentration (M)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>1.0</td>
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<tr>
<td>K$^+$</td>
<td>30</td>
<td>30</td>
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</tr>
<tr>
<td>Cl$^{-}$</td>
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<td>16</td>
<td>16</td>
<td>78</td>
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<tr>
<td>H$_2$O</td>
<td>4055</td>
<td>4056</td>
<td>4049</td>
<td>3925</td>
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</table>
the ions belonging to the cluster (either ion 4A or 4B), was found successful in reproducing the experimental diffraction patterns of the hydrated cluster (in those simulations each of the Mg$^{2+}$ ions remained close to its starting position). Thus, simulations $4\text{MgA}$ and $4\text{MgB}$ are analyzed here for evaluating the binding behavior of monovalent ions to the deep groove of the LE motif that is partially filled with Mg$^{2+}$ ions.

Results

Stability of the trajectories

As shown before, the structural integrity of LE is preserved during the 10 ns of the $4\text{MgA}$ and $4\text{MgB}$ trajectories due to the presence of the deep groove bound Mg$^{2+}$ ions. Without divalent ions and at a physiological concentration of KCl (NoMg), the LE is more flexible (Table 2 and Figure 2) and a partial opening of the trans Watson–Crick/Hoogsteen A73-U103 pair has been reported (similar opening events were observed for the symmetric U77-A99 pair in independent simulations conducted on LE in the absence of Mg$^{2+}$ ions).

At a higher monovalent ion concentration (NoMg1M), the overall mobility of the duplex is reduced to a level similar to the one observed in the $4\text{MgA/B}$ simulations as reflected by the calculated hydrogen bond percentages (HB%; Table 2) and per-residue $B$-factor values (Figure 2) which reveal a significant degree of stabilization induced by the high concentration of monovalent ions.

K$^+$ ion binding regions

In the following, because of the irregular shape of the ion binding locations, a distinction is made between K$^+$ binding “regions” corresponding to electronegative zones to which monovalent ions are attracted and K$^+$ binding “sites” associated with maxima (given by the coordinates) in the ion density profiles calculated by the PEAKMAX program of the CCP4 (see Computational Methods section).

The $4\text{MgA}$ and $4\text{MgB}$ simulations

In these two simulations, the Mg$^{2+}$ ions remain close to their starting X-ray positions. Due to electrostatic repulsions, K$^+$ ions do not penetrate the parts of the deep groove occupied by the divalent ions. Nevertheless, two main and one minor K$^+$ binding regions common to the $4\text{MgA/I}$ and NoMg simulations were found at locations in the deep groove devoid of Mg$^{2+}$ ions (K1, K2, and K3, respectively; see Figure 3 and Table 3).

The K1 region has an elongated shape and is facing the (G)O6/N7 atoms of the G72-A104 pair. On average, it is occupied by 0.8 ion in simulation $4\text{MgB}$ ($\sim$0.1 in $4\text{MgA}$; see Table 4). The K2 region, which contains $\sim$0.5 ion, has a similar shape and is located in front of the (G79)N7/O6 atoms. Additionally, K2 is close to the O4 atom belonging to the G96oU80 pair. Interestingly, as a result of the proximity of a weaker K$^+$ binding region (K3) involving the participation of the O6 atom of the dangling residue G81, the number of ions contacting the (U80)O4 atom is particularly high ($\sim$0.8; see Table 3).

Compared to the other two regions, K3 appears as relatively minor, since it is associated with densities lower than the ones calculated for the K1 and K2 regions and, although at contact distance from the GoU pair, K3 is not exactly located in the deep groove of the helix. Still, the K2 and K3 regions indicate that GoU pairs present good binding opportunities for monovalent ions.

Figure 2. Superimposition of the curves representing the per-residue $B$-factors calculated over the last 10 ns of trajectories NoMg, NoMg1M, and $4\text{MgA/B}$ (average values for the $4\text{MgA}$ and $4\text{MgB}$ trajectories are shown) along with the experimental values. In the secondary structure, non-Watson–Crick pairs are drawn in red. Note that the absolute values of the calculated and experimental $B$-factors cannot be directly compared since, in the crystal, restricted motions of the solute around its lattice position (clearly seen at the extremities) do occur while for the calculated values a fitting procedure was used.
The NoMg simulation

In the NoMg simulation K\(^+\) ions are free to penetrate into the deep groove. As a result, seven well defined ion-binding regions can be characterized (Figure 3). Beside regions K1, K2, and K3 that are occupied by a similar number of ions in simulations 4MgA/B and NoMg (Table 4), high ion density regions are located close to each of the original Mg\(^{2+}\) positions (1, 2, 3, 4A, and 4B; see Figure 1). Hence, the same naming convention will be used for the Mg\(^{2+}\) binding sites and the K\(^+\) binding regions (however, we will refer to region 4 when discussing the K\(^+\) densities close to the Mg\(^{2+}\) ions 4A and 4B).

Region 1, which is occupied by \(\approx 0.8\) ion, is associated with a Watson–Crick type GpG step and shows an elongated density spanning the internucleotide space between the N7/O6 atoms of the G105pG106 step (Table 4). On the other hand, region 2 is located in the deep groove of the non-Watson–Crick G75pG76 step and is composed of two well separated and almost spherical ion density spots which will be named 2a and 2b (see Figure 3). Here, the K\(^+\) ions, while avoiding (G75)O6, interact essentially with the (G75/G76)N7 and (G76)O6 atoms. The occupancies of these sites designate 2a as the primary (\(\approx 0.9\)) and 2b as the secondary (\(\approx 0.4\)) binding site (Table 4).

Table 2. Hydrogen bonds as seen in the LE crystal structure and average values (in Å) as well as hydrogen bonding percentages (HB\%) calculated over the last 10 ns of the 4MgA, 4MgB, NoMg1M, and NoMg trajectories

<table>
<thead>
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<th>Hydrogen bonds involved in base-pair interactions</th>
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<tr>
<td>Crystal</td>
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<td>(70)C=G(106)</td>
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<td>N3=H-N1</td>
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<td>N4=H-O6</td>
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<td>(71)C=dG(105)</td>
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<tr>
<td>N4=H-O6</td>
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<tr>
<td>(72)G-A(104)</td>
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<tr>
<td>N2=H-N7</td>
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<tr>
<td>O2'=H-N6</td>
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<tr>
<td>(73)A-U(103)</td>
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<tr>
<td>N6=H=O2</td>
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<tr>
<td>(74)U-G(102)</td>
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<tr>
<td>O4=H-N1</td>
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<td>O4=H-N2</td>
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<td>(75)G-A(101)</td>
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<td>O6=H-N6</td>
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<td>(76)G-G(100)</td>
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<td>N1=H-O6</td>
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<tr>
<td>(77)U-A(99)</td>
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<tr>
<td>N3=H=N7</td>
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<tr>
<td>O2'=H-N6</td>
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<tr>
<td>(78)A-G(98)</td>
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<td>N6=H=N3</td>
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<td>N6=H=O2</td>
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<td>(79)G=C(97)</td>
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<td>N2=H=O2</td>
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<tr>
<td>O3=H-N1</td>
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<tr>
<td>N3=H=O6</td>
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</tbody>
</table>

**Intra-strand hydrogen bonds**

| (72)G--A(73) | O2'=H=O4′ | 2.1 | 2.1 (60) | 2.2 (53) | 2.6 (10) |
| (98)G--A(99) | O2'=H=O4′ | 2.0 | 2.1 (65) | 2.1 (65) | 2.3 (47) |

HB\% are defined as the total number of hydrogen bonds established during a single trajectory divided by the total number of configurations analyzed. HB\% lower than 80% are in bold.

* The values obtained for simulations 4MgA and 4MgB have been averaged.

* For the crystal structure, the chosen orientation of the 2'-OH groups is such that it minimizes the intra-strand hydrogen bond distance.

The NoMg simulation

In the NoMg simulation K\(^+\) ions are free to penetrate into the deep groove. As a result, seven well defined ion-binding regions can be characterized (Figure 3). Beside regions K1, K2, and K3 that are occupied by a similar number of ions in simulations 4MgA/B and NoMg (Table 4), high ion density regions are located close to each of the original Mg\(^{2+}\) positions (1, 2, 3, 4A, and 4B; see Figure 1). Hence, the same naming convention will be used for the Mg\(^{2+}\) binding sites and the K\(^+\) binding regions (however, we will refer to region 4 when discussing the K\(^+\) densities close to the Mg\(^{2+}\) ions 4A and 4B).

Region 1, which is occupied by \(\approx 0.8\) ion, is associated with a Watson–Crick type GpG step and shows an elongated density spanning the internucleotide space between the N7/O6 atoms of the G105pG106 step (Table 4). On the other hand, region 2 is located in the deep groove of the non-Watson–Crick G75pG76 step and is composed of two well separated and almost spherical ion density spots which will be named 2a and 2b (see Figure 3). Here, the K\(^+\) ions, while avoiding (G75)O6, interact essentially with the (G75/G76)N7 and (G76)O6 atoms. The occupancies of these sites designate 2a as the primary (\(\approx 0.9\)) and 2b as the secondary (\(\approx 0.4\)) binding site (Table 4).

The ion densities associated with region 4 in front of the (G102)N7/O6 and (A101)N7 atoms are similar to those described for region 2. Again, two spherical density spots (4a and 4b; Figure 3) can be distinguished and site 4a, that is close to the N7 atom, has a higher occupancy (\(\approx 0.9\)) than site 4b facing the O6 atom (\(\approx 0.4\)).
Table 3. Number of K$^+$ ions contacting the deep groove O4, O6, and N7 atoms, the OR and OS anionic oxygen atoms of the phosphate groups, and the guanine bases averaged over the last 10 ns of the four 4MgA, 4MgB, NoMg1M, and NoMg trajectories.

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<tr>
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<th>NoMg</th>
<th>4MgA/B</th>
<th>NoMg1M</th>
<th>NoMg</th>
<th>4MgA/B</th>
<th>NoMg1M</th>
<th>NoMg</th>
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<th>4MgA/B</th>
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<td>K(3)</td>
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<td>0.3</td>
<td>0.1</td>
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</table>

A contact is defined by an ion to RNA atom distance below 3.5 Å. The atoms associated with a same K$^+$-binding region (see columns R in italics) are shaded. The ones involved in direct contacts with Mg$^{2+}$ ions are marked with an asterisk. Values equal to or higher than 0.3 are in bold.

For the guanine bases, a contact is defined by an ion to base atom distance below 3.5 Å. Thus, the numbers given in these columns are not equal to the sum of the individual contributions calculated for the O6 and N7 atoms.

The numbers given in this line do not correspond to the sums of the individual atomic (or guanine) contributions as some overlap between adjacent binding sites is expected.
Region 3 is occupied by \( \approx 1.3 \) ion and is also divided into two main and one minor ion density zones. The first zone is located in front of the (G98)N7/O6 atoms while the second is close to the Watson–Crick edge of the (G)O6 atom (Figure 3). A third relatively small spot is seen in front of (G)O6. Yet, since this latter region is less regular than regions 1 and 2, it is also more difficult to dissect into spherical regions and will, therefore, not be described in further detail. Interestingly, region 3 is equivalent by symmetry to region K1 and their ion binding profiles are roughly similar, thus assessing the quality of the conformational sampling achieved by the simulations. Next to these regions, \( K^+ \) ions form only fleeting contacts with the oxygen atoms of the phosphate groups. Hence, no strong \( K^+ \)-binding region is associated with them at this ionic concentration.

As a particular feature of simulation NoMg, it can be noted that the partial opening of the \( \text{trans} \) Watson–Crick/Hoogsteen A73·U103 pair leads to a better accessibility of (U103)O4 thus inducing the formation of a secondary binding site occupied by \( \approx 0.5 \) K\(^+\) atom (Table 3).

### The NoMg1M simulation

The increase in the concentration of added KCl (from 0.2 M to 1.0 M) does not qualitatively modify the repartition of the \( K^+ \)-binding sites observed in simulation NoMg (Figure 3). On the average, the total number of ions contacting the 11 guanine bases is not significantly altered (from 6.4 to 6.9; see Table 3). However, two exceptions can be noted. First, K3 has now to be considered as a primary binding region (\( \approx 1.0 \) ion is touching G81) and a significant augmentation (from 0.9 to 1.4) of the number of ions contacting (U80)O4 is calculated. Hence, it is expected that a further increase of the concentration of KCl would lead to a complete dehydration of that atom. Secondly, the sites 2b and 4b associated with (G76)O6 and (G102)O6 disappear at the benefit of sites 2a and 4a which are now fully occupied by a \( K^+ \) ion. In contrast to the guanine bases, the total number of ions contacting the OR as well as the OS anionic phosphate oxygen atoms increases roughly by 1.0 (Table 3) with the higher KCl concentration and, at the same time, a new density associated with ions bridging the (U74)OR and (A99)OR atoms emerges at the entrance of the deep groove (shown by blue dotted lines in Figure 3). With this single exception,

### Table 4. Occupancy of the \( K^+ \)-binding regions

<table>
<thead>
<tr>
<th>Region</th>
<th>4MgA/B</th>
<th>NoMg1M</th>
<th>NoMg</th>
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<tr>
<td>K3</td>
<td>0.4/0.4</td>
<td>0.7</td>
<td>0.3</td>
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<td>0.6/0.5</td>
<td>0.9</td>
<td>0.6</td>
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<td>1.0</td>
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<tr>
<td>1</td>
<td>-</td>
<td>0.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* The occupancy values of the “a” and “b” sites belonging to region 2 and 4 in simulation NoMg are given in parenthesis.

Figure 3. Ion-binding regions associated with the LE motif calculated from the last 10 ns of trajectories 4MgA and 4MgB (averaged to a 1:2 ratio; see Ref. 47), NoMg, and NoMg1M. For 4MgA/B, the Mg\(^+\) and K\(^+\) densities are shown in yellow and violet, respectively. For NoMg and NoMg1M, the K\(^+\) densities are shown in green and red, respectively. A density level of 0.5 e/Å\(^3\) is used. In the NoMg1M panel, a secondary K\(^+\) site bridging the OR atoms of U74 and A99 is marked by dotted yellow lines.
phosphate groups do not constitute good grips for the formation of well defined K\(^+\)-binding sites.

More generally, the total number of ions contacting the base atoms rises by 1.0 (from 7.0 to 8.0; see Table 5), while the number of ions contacting the backbone atoms increases by 2.7 (from 5.5 to 8.2) which implies that, at the concentration of 0.2 M of added KCl, most of the deep groove monovalent binding sites are already saturated. Thus, the augmentation of 3.7 in the number of ions contacting the entire duplex (from 10.8 to 14.5) results essentially from the saturation of region 3 and from additional contacts made by the ions with backbone and shallow groove atoms (the number of ions contacting both the base and backbone remains constant). Indeed, a K\(^+\)-binding site occupied by \(\approx 0.2\) K\(^+\) ion is located in front of the N1 atom of A99 (site S1, see Figures 3 and 4), indicating that at still higher concentrations of monovalent ions, the accessible Watson–Crick edges of adenine bases may represent significant ion-binding locations.

The presence of the Mg\(^{2+}\) ions in the 4MgA/B simulations leads to a diminution of the number of ions binding to the base and of those binding to the backbone atoms, since almost all Mg\(^{2+}\) ions contact an OR atom either directly or through water-mediated interactions (Table 5).

**Guanine ion-binding sites**

As detailed above, ion-binding sites refer to peaks located in high ion density regions. Given the irregular shape of these regions, usually several peaks of various heights can be associated with them. This can be clearly seen for the regions 1, 3, K1, and K2 in simulations NoMg and NoMg1M (Figure 3) while in simulation NoMg, as already described, the regions 2 and 4 can be split without ambiguity into two sites (a and b). A compilation of all K\(^+\)-binding sites indicates that they are almost all associated with the N7/O6 atoms of guanine bases and reside either in the plane of the base or bridge two adjacent nucleotides (Figure 5). Thus, it appears that the Hoogsteen edge of guanine bases is a major component of monovalent ion-binding sites, even though the precise location of the ion around the base is context dependent.

**Dynamical behavior of the bound K\(^+\) ions**

In this section, the residence times of K\(^+\) ions to specific regions will be estimated by using a graphical representation that appears more apt to apprehend the complexity of the binding phenomena (Figure 6), since such representations allow us to deal in a more flexible way with interruption times that find their origin in ions making “short” and “long” excursions outside of their binding regions.

Among all, regions 2 and 4 are the most efficient in retaining monovalent ions (Figure 6). Indeed, the same ion (shown in blue) occupies site 4a for more than 9 ns despite some vacancies observed during that time (note that the ion is not replaced by any other during those periods). The five other regions are characterized by binding of a much larger number of ions which exchange relatively
frequently (generally more than six over the 10 ns trajectories). Accordingly, the residence times of the ions in those regions are much shorter.

The particular shape of regions 2, 3, and 4, and the associated occupancy values (larger than 1.0; see Table 4) indicate that these regions are sometimes simultaneously occupied by two K\(^+\) ions separated by a small distance. Indeed, in simulation NoMg, “ion pairs” are clearly observed for regions 2 and 4 (Figure 6) which both are split into two spherical zones, one of higher and one of lower occupancy, as well as for the less regular region 3 (Figure 3). The simultaneous occupation of one area by two ions occurs mainly in region 2 between 8 ns and 10 ns and in region 4 between 3 ns and 8 ns. Although the minimal distance of approach that has been recorded is of \(\approx 3.1\) Å, the average distances between K\(^+\) ions occupying these regions are of \(4.3(\pm 0.6)\) Å and \(3.9(\pm 0.4)\) Å, respectively, which is close to the first peak of the K\(^+\)···K\(^+\) distribution function (4.1 Å) calculated for all the K\(^+\) ions present in the NoMg and NoMg1M simulations (note that short M\(^{2+}\)···M\(^{2+}\) distances are not rare in biological systems\(^{59}\)). Thus, regions 2 and 4 seem to attract monovalent ions in a site bound way, since they are characterized by the binding of at most two K\(^+\) ions simultaneously among which one of them displays very long residence times (Figure 6), while K\(^+\) ions bind in a more diffuse way and with shorter residence times to the other regions.

No clear difference in the dynamical behavior of the monovalent ions between simulations NoMg and NoMg1M is observed. In both simulations, regions 2 and 4 are able to keep monovalent ions for very long times while the other sites form more fleeting contacts. However, sites K2 and K3 located at the end of the duplex show a higher occupancy in NoMg1M compared to NoMg.

**Occupation of ion-binding regions by K\(^+\), Mg\(^{2+}\), and water molecules**

As noted above, all the Mg\(^{2+}\)-binding sites detected in the crystal structure possess a close-by K\(^+\)-binding region. Still, with the exception of

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**Figure 6.** Occupation by K\(^+\) ions of the seven ion-binding regions calculated for the NoMg and NoMg1M simulations. The ions displaying the highest occupancies are shown in blue, those among the first six which display the lowest occupancies are colored in yellow (see scale). Black stripes indicate that more than six different ions occupy a given region and white stripes mark periods where no ions are present.
region 3 where the Mg$^{2+}$ ion establishes a direct contact with a (G)O6 atom, these sites do not overlap. In fact, the K$^+$ ions always contact the base atoms directly while the Mg$^{2+}$ ions contact them through water-mediated interactions.\(^41\,47\) Thus, the K$^+$ sites are intercalated between the base and the Mg$^{2+}$ sites. More precisely, for regions 1, 2, and 4 in simulation NoMg1M the K$^+$ ions are located at distances ranging from 1.6 Å to 2.9 Å from the Mg$^{2+}$ positions taken from simulations 4MgA/B (Figure 7), which roughly corresponds to the distance separating the divalent ion from a water molecule belonging to its first coordination sphere (=2.1 Å).\(^50\) Additionally, the K$^+$ ions are located at a distance of ≈2.7 Å from the (G)N7/O6 atoms, which is close to the equivalent distance involving water molecules (≈2.8 Å).

Although all the regions are occupied by K$^+$ ions, some vacancies can be seen in Figure 6 during which it is observed that water molecules enter the binding regions. Furthermore, some regions are large enough to accommodate simultaneously a K$^+$ ion and a water molecule. It is quite clear that while sites 2a and 4a are almost entirely occupied by a K$^+$ ion at 0.2 M of added KCl, the close-by sites 2b and 4b are only partially occupied by ions, whereas during the remaining time they are filled by water molecules. Fractional occupancies are also noticeable in simulation NoMg for the regions K2 and K3 located at the end of the duplex.

Discussion

The LE deep groove is highly ionophilic...

For the eubacterial LE fragment, seven deep groove K$^+$-binding regions with occupancies higher than 0.5 have been characterized (Figure 3). The ions occupying these regions are essentially involved in direct contacts with the O6/N7 atoms of guanine bases and less frequently with the (A)N7 and (U)O4 atoms of GpA and GpU steps indicating, in agreement with a recent survey of the nucleic acid database,\(^48\) that the highly electronegative Hoogsteen edge of guanine base is a major component of monovalent ion-binding sites (Figure 5). Not surprisingly, the major/deep groove edge of guanine base is also recognized as being the central nucleic acid binding location for divalent ions.\(^13\,17\,51\,53\)

Accordingly, it is found that the deep groove edge of the G96oU80 pair is a significant ion-binding region. Indeed, the deep groove of wobble GoU pairs is one of the most electronegative among non-Watson–Crick pairs. Interestingly, in our simulations, the K$^+$ ions assigned to region K2 avoid direct contacts with the (G96)N7/O6 sites in favor of the O6 and O4 atoms belonging to the G79pU80 step (Figure 8). A related interaction scheme is observed in a RNA crystal structure (NDB: pr0037)\(^54\) in which two Cs$^+$ ions, interacting with a tandem of GoU pairs, occupy sites which are strikingly similar to sites K2 and K3.\(^8\) Elsewhere, a Tl$^+$ ion is contacting a GoU pair in a different manner (it contacts the N7/O6 sites of the GoU pair and the O4 atom of an adjacent A·U pair).\(^8\) Thus, even though the precise coordination of the monovalent ions is definitely modulated by the structural context, the electronegative regions associated with wobble GoU pairs and GpU steps have to be considered as recurrent ion binding locations.

Shallow groove ion-binding sites generally appear secondary for RNA duplexes.\(^22\,23\) In the present simulations the Watson–Crick edge of the
adenine base involved in the trans Watson–Crick/ Hoogsteen U77-A99 pair is one of these minor sites starting to be occupied only at a high concentration of monovalent ions. With regard to the backbone, although a significant number of contacts is recorded between K\(^+\) ions and phosphate groups, the ions essentially interact with them in a diffuse manner. Still, at the higher ionic concentration, a K\(^+\)-binding site bridging two OR atoms from separate strands was located at the entrance of the deep groove (Figure 3). Thus, once the deep groove is filled, secondary sites associated with phosphate groups can emerge at locations where the deep groove narrows (note that Mg\(^{2+}\) ions bridging phosphate groups in a similar manner were observed elsewhere).

As an outcome, since most of the K\(^+\) ions condense to the deep groove of LE (see Figure 3), this region appears highly ionophilic as also shown in a recent study conducted on the same LE motif. Indeed, the ion uptake ability of the LE deep groove is larger than that of regular RNA duplexes, for which it is calculated to be close to 0.2 K\(^+\) ions per A·U and C·G pairs. This is most certainly related to the accretion of non-Watson–Crick pairs forming this motif which leads to a deep groove that is narrower and, consequently, more electronegative than that of Watson–Crick duplexes.

...and can accommodate monovalent and divalent ions...

Although monovalent ions compete with divalent ions for binding to RNA molecules, structural data indicating in which manner monovalent ions may occupy Mg\(^{2+}\)-binding sites are limited. In an RNA structure obtained at 1.5 Å resolution (NDB: pr0037), a K\(^+\) ion is found in direct contact with the RNA and at 2.0 Å from a hydrated Mg\(^{2+}\) ion. Similarly, in a DNA structure obtained at 1.2 Å resolution (NDB: bd0054), two Ti\(^+\) ions with Mg\(^{2+}\)-Ti\(^+\) distances of 2.0 Å and 2.5 Å were detected. All these sites were refined with occupancy factors below one and it was proposed that monovalent ions able to occupy the position of one of the inner sphere-coordinated water molecules represent a general theme at sites which can accommodate both monovalent and divalent ions.

The present simulations, where the K\(^+\)-binding sites are found at distances ranging from 1.6 Å to 2.9 Å from the Mg\(^{2+}\)-binding sites, intercalated between them and the base atoms (Figure 7), support the view that each Mg\(^{2+}\)-binding site possesses a close-by K\(^+\)-binding region and that these regions are roughly located at the same distances from the divalent ion as the water molecules belonging to its first hydration shell. This may be true at least for the Mg\(^{2+}\) ions which contact the base atoms through water-mediated interactions (probably the most frequently observed binding scheme for the “hard” Mg\(^{2+}\) ions). Yet, monovalent and divalent cations are most certainly never so close in space. When such distances are observed, they are due to crystallographic disorder and fractional occupancies.

Another issue of importance is related to the occupancy of monovalent ion-binding sites by charged chemical groups belonging to proteins or drugs. Indeed, in the LE/L25 complex the –NH\(^{3+}\) head of Lys14 binds to the G79pU80 step close to the ion-binding region K2 (Figure 8). Noteworthy, a GpU step associated with one or two wobble GoU pairs is found in many sequences of the eubacterial 5S rRNA LE motifs, probably in order to similarly accommodate the ammonium group of a lysine residue coming from an associated ribosomal protein. Hence, monovalent ion-binding sites represent good anchor points for ammonium groups of proteins and drugs.

...with long residence times...

At the two chosen ionic concentrations, regions 2 and 4 located close to the symmetry center of the LE motif (the G75-A101 pair; see Figure 9) are occupied by K\(^+\) ions which display exceptionally long residence times while the more distant regions

Figure 8. The GpU ion-binding regions. (Right) Ion-binding regions K2 and K3 which contact the (U80)O4 atom calculated from simulation NoMg1M. (Left) View extracted from the crystal structure of the complex between the ribosomal protein L25 and the 5S rRNA LE motif showing the –NH\(^{3+}\) group of Lys14 located close to the monovalent ion-binding sites detected at the same GpU step in the NoMg and NoMg1M simulations.
(1, 3, and K1-3; see Figure 9) attract K\(^+\) ions in a more diffuse manner.

In that respect, a parallel can be established between K\(^+\) and Mg\(^{2+}\)-binding sites, since regions 2 and 4 have been recognized, based on theoretical and structural data, as main binding locations for hydrated Mg\(^{2+}\) ions while binding to regions 1 and 3 is reported to be incidental. For instance, it was found that a hexahydrated Mg\(^{2+}\) ion is more mobile when it binds to a Watson–Crick (region 1) rather than to a non-Watson–Crick GpG step (region 2). The same binding behavior is observed here for K\(^+\) ions. Although it is clear that mono- and divalent cations are attracted to regions of high electronegative potential, it was shown for hydrated Mg\(^{2+}\) ions that the specificity in binding is modulated by the structural complementarity existing between the hydrated ion and the hydrophilic atoms lining up the binding pockets. Still, no major difference associated with the contacts established between the K\(^+\) ions and the guanine bases of both GpG steps (which exhibit very similar stacking patterns; see Figure 7) can be noted. However, as for Mg\(^{2+}\) ions, the indirect interactions established by the K\(^+\) ion with the environment surrounding these GpG steps may reside at the origin of the observed differences in binding specificity. For example, the arrangement of hydrophilic atoms in both grooves is different and the deep groove of region 2 is narrower than that of region 1 leading to a more electronegative binding pocket (note also that the C71 residue is devoid of phosphate group; see Figure 7).

Thus, the differences in the dynamical behavior of mono- and divalent ions estimated from MD simulations strongly suggest that regions 2 and 4 are the main ion-binding regions (it has also been recently emphasized that these regions display the highest electronegative potential). Therefore, Mg\(^{2+}\) ions associated with these regions may be more difficult to displace by K\(^+\) ions. For the other electronegative sites of the deep groove which attract mono- and divalent ions in a more diffuse way, K\(^+\) ions may enter in competition with Mg\(^{2+}\) at a much lower concentration of added KCl.

...in a symmetric manner

Interestingly, the mono- and divalent ion-binding regions 2 and 4 as well as K1 and 3 are “symmetric” with respect of the central C75-A101 pair of the motif (Figure 9) although, as shown by the crystal structures of the unbound and complexed LE motif, regions 2 and 4 attract hexa- and pentahydrated ions, respectively. This feature extends the “pseudo-symmetry” of the eubacterial LE motif characterized by the two similar submotifs that are shown in Figures 1 and 9. Indeed, the deep groove ion-binding regions 2 and 4 are located close to an area that displays a specific shallow groove pattern essential for the binding of the ribosomal proteins L25 and TL5. However, this region (involving a water-mediated G-A and two bifurcated G-G and U-G pairs) is characterized by a weak hydrogen bonding pattern (see Figures 7...
and 9). In order to stabilize this very specific and fragile shallow groove protein binding motif, the association of divalent ions to regions 2 and 4, as observed in the available crystal structures, is certainly essential. Binding of divalent ions to region 3 (or to its symmetric site K1), when it is observed, is associated with lattice interactions and, thus, probably not of great importance for the structure and function of the eubacterial 5 S rRNA LE motif.

Hence, it can be speculated that the LE motif and the associated symmetric deep groove ion-binding regions 2 and 4 constitute the functional unit that is recognized by ribosomal proteins. It is expected that in vivo, these ion-binding regions are occupied by Mg$^{2+}$ ions while the other locations are occupied by K$^+$ (or other monovalent) ions. However, Mg$^{2+}$ titration experiments by NMR reveal that these ions appear to be dispensable for stabilization of the bound RNA structure and for stable binding of the protein to its cognate RNA ligand.

The effect of high monovalent ion concentrations

A high concentration of monovalent ions is generally able to stabilize the structure of RNA systems and to initiate the formation of correct folds. Such an effect was reported for the eubacterial 5 S rRNA LE for which a melting temperature increase of $\approx 23^\circ$C was found when going from a 0.1 M to a 1.0 M NaCl buffer. This effect is especially important for LE, since it is at least three times greater than the one observed for other RNA duplexes including the eukaryotic 5 S rRNA LE motif. In the present simulations conducted at 0.2 M and 1.0 M of added KCl, although a stabilization of LE is observed, the occupancy of the deep groove ion-binding regions is comparable in both simulations. Thus, even at 0.2 M of added KCl, these regions are close to saturation and an increase in concentration leads solely to a further partial dehydration of the phosphate groups (on average, an increase of 0.08 K$^+$ ion per phosphate group is noted). From a dynamical point of view, the difference in the calculated residence times of K$^+$ ions at low and high KCl concentrations is also difficult to quantify (Figure 6). Concentrations of added KCl below 0.2 M are certainly necessary to desaturate regions 2 and 4.

Comparisons with Brownian dynamics simulation results

The detection of potential ion-binding sites is a major issue of simulation methods and Brownian dynamics (BD) techniques have been used with some success in order to locate Mg$^{2+}$-binding sites associated with various RNA structures. For the eubacterial LE motif, the use of probes of different radii led to the placement of seven divalent ions. Among those, five were located at distances ranging from 0.7 Å to 2.7 Å from the crystallographic Mg$^{2+}$ ion positions and two occupied the deep groove at the level of the G96oU80 pair. A closer examination of the MD, BD, and crystallographic ion-binding sites revealed further that all the experimental and calculated sites cluster into the already described seven ion-binding regions (however, site K1 that is symmetrically equivalent to site 3 and the marginal site K3 remained undetected). Interestingly, the BD study described also the site K2 close to the G96oU80 pair that can accommodate the (L25:Lys14)–NH$_3^+$ group (see Figure 8).

Still, BD simulations are not meant to discriminate between monovalent and divalent ion-binding sites, since the stereochemistry of the hydrated Mg$^{2+}$ ions is not accounted by the simple spherical probes used (even if they carry a +2 charge). Indeed, detecting specific Mg$^{2+}$-binding sites is a difficult exercise since these ions are most often penta- or hexahydrated and bind to RNA through water-mediated and/or direct interactions. Although divalent ions (like monovalent ions) are attracted to electronegative pockets, the specificity of binding is modulated in that case by the structural complementarity existing between the hydrated ion and its binding site.

As shown here, atomistic MD simulations can reliably detect monovalent ion-binding sites, a feature which may be of importance in drug-design studies, since at least some monovalent ion-binding sites represent good anchor points for ammonium groups belonging to proteins and drugs. In this perspective, one of the determining advantages of MD over BD simulations resides in their ability to discriminate efficiently between strong and weak ion-binding sites and to take into account the inherent flexibility of the associated binding pockets.

Sampling and force-field issues

The 10 ns sampling and $\approx 1.5$ ns equilibration trajectories that were accumulated for each of the four simulations described here, although quite long with respect to current protocols, are still very short compared to the time scales of a large number of important biological phenomena. For example, the desolvation of hexahydrated Mg$^{2+}$ ions and the formation of inner sphere complexes take microseconds to occur and cannot be reproduced by present MD simulations. Therefore, the simulations described in the earlier study of the LE motif and also discussed here, focused only on the structural and dynamical characterization of hydrated divalent ions occupying their well-defined crystallographic positions. On the other hand, the intrusion of monovalent ions into the RNA first hydration shell can be much more easily addressed given the different time scale on which these ions are dehydrated (well below 100 ps for Na$^+$ and K$^+$). Therefore, here and in other simulations, the ions were systematically placed in a manner that none of them was closer than 8 Å from any RNA atom (see Computational Methods).
in order to ensure that the ions would find their “equilibrium” positions by themselves. Indeed, in all four simulations, it was observed that the ions did penetrate the LE deep groove in a reproducible manner and occupy electronegative pockets located essentially in front of the N7 and O6 atoms of guanine bases. The fact that similar ion-binding sites were occupied by K\(^+\) ions in all four simulations (see Figure 3) suggests that the sampling time was sufficient with respect to that property. The reliability of the detected K\(^+\)-binding sites is further assessed by the fact that, as described above, most of them well match monovalent binding sites observed in other crystallographic structures of nucleic acids.\(^{17,48}\) At last, force-field problems are smaller for monovalent than divalent ions, since the former are less affected by the neglect of polarization and charge transfer effects (see also discussion in Ref. 36).

**About long residence times and “strong” binding sites**

In these simulations, three main types of monovalent ion binding behavior during which the ions are partially dehydrated were observed: (i) binding to a given site with long residence times; (ii) binding to specific sites in a more diffuse way characterized by shorter residence times and (iii) delocalized interactions with the RNA. As mentioned above, the K\(^+\) ions equilibrated around the RNA in a manner that was reproducible and not biased by the initial conditions. Indeed, in MD simulations as in real systems, the ions are influenced at each time step by all electrostatic and van der Waals interactions originating from every particle present in the simulation box, namely RNA atoms, water molecules, K\(^+\), and Cl\(^-\) ions. Hence, the ions are attracted by sites in a stronger or weaker manner depending, among other factors, on the electronegative character, shape, and surrounding of these sites and thus all the energetic contributions such as the desolvation cost of the ions and their binding sites along with their thermal energy are taken into account. For electronegative locations that are exposed to the solvent, such as those close to the OS atoms, the thermal energy of the ions is generally sufficient to favor rapid exchange with neighboring solvent molecules, while for more buried sites like those located in the deep groove, the exchange rate of the bound ions is much lower, especially for the ions associated with the central sites 2 and 4. Hence, MD simulations allow us to discriminate between “diffuse”, “weak”, and “strong” binding of monovalent ions. Yet, “strong binding” is a relative concept, since it relies on the RNA architecture and local foldings. For example, it is probable that a K\(^+\) ion that interacts directly with three buried phosphate groups in an rRNA fragment\(^{14}\) has a larger association constant than those located in the deep groove of the 5S rRNA LE. Other methods like those that directly estimate the ion binding free energy contributions could be used to classify such monovalent ion-binding sites in a more definite manner.\(^{2,14}\)

**Summary and Conclusions**

On the basis of recent UV melting experiments, it was suggested that one or at most two Mg\(^{2+}\) ions bind strongly to the eubacterial LE motif and are required for its structural stability.\(^{43}\) Still, that study did not give any clue as to which of the five Mg\(^{2+}\) ions detected by crystallography\(^{41}\) is to be considered as structurally important. The MD simulations conducted on LE in the presence and absence of Mg\(^{2+}\) ions\(^{47}\) and at two concentrations of added KCl allow us to propose a refined view of the ion binding features of the LE motif that extends those obtained by crystallography\(^{41,56,57}\) and other biophysical methods:\(^{9,42,44,46,56,62}\)

1. Seven K\(^+\)-binding locations are present in the LE deep groove, which thus appears more ionophilic than the equivalent groove of regular RNA duplexes.\(^{22,23}\)
2. At a concentration of 0.2 M of added KCl, the ions essentially condense to the deep groove base atoms while no well defined ion-binding sites are associated with the shallow groove and backbone atoms. At the higher concentration (1.0 M), shallow groove and backbone ion-binding sites start to emerge.
3. The highly electronegative Hoogsteen edge of guanine bases is a major component of ion-binding sites, since K\(^+\) ions occupying the deep groove interact mainly with the O6/N7 atoms of guanine bases at GpG, GpA and GpU steps. Wobble GoU pairs are also good ion-binding locations even though the precise coordination of the monovalent ions to the guanines or the GoU pairs appears context dependent.
4. Some ion-binding regions can accommodate mono- and divalent ions since the Mg\(^{2+}\) ion-binding locations 1, 2, and 4 possess a close-by K\(^+\)-binding region with a Mg\(^{2+}\)⋯K\(^+\) distance (=1.6–2.9 Å) close to the Mg\(^{2+}\)⋯Ow distance (=2.1 Å) that is related to inner-sphere water molecules.
5. Four (K1, 2, 3, and 4) out of the seven K\(^+\)-binding sites are organized in a symmetric manner with respect to the central C75-A101 pair of the stack of seven non-Watson–Crick pairs constituting the LE and, therefore, match the “pseudo-symmetry” of this motif (site 2 is symmetric to 4 and K1 is symmetric to 3 with respect to the central G:A pair).
6. K\(^+\) ions bound to the regions 2 and 4 display very high residence times and, consequently, do not exchange frequently, while K\(^+\) ions bind to the other regions in a more diffuse way. Hence, regions 2 and 4 can be considered as the principal monovalent ion-binding locations of the LE motif.
(7) Monovalent ion-binding sites can accommodate the –NH$_3^+$ group of lysine residues and most certainly also charged groups belonging to drugs like aminoglycoside groups.

From these observations, it can be inferred that the occupation of sites 2 and 4 is required in order to stabilize the particular arrangement of shallow groove hydrophilic atoms that are involved in the specific recognition of ribosomal proteins associated with this “pseudo-symmetric” eubacterial 5 S rRNA LE motif. In vivo, these ion-binding locations (2 and 4) are thus most probably occupied by Mg$^{2+}$ ions while the others are engaged in the binding of monovalent ions.

Indeed, with the joint development in experimental techniques and MD simulation methods, the picture of the role played by monoatomic ions in the stabilization processes of RNA systems has gained in precision but is also becoming more intricate, since it involves the participation of several ionic species that compete with each other for electronegative binding sites. Clearly, divalent but also monovalent ions are of primordial importance for many biochemical processes since it is likely that both ion types contribute to the charge neutralization and stabilization mechanisms of RNA molecules through site specific and delocalized or diffuse interactions. The inescapable conclusion is that both monovalent (K$^+$) and divalent (Mg$^{2+}$) ions along with water molecules contribute to the activity of RNA systems.

**Computational Methods**

Four 11.45 ns MD simulations based on the 1.5 Å resolution crystal structure of the 5 S rRNA LE motif (NDB code: url064; PDB code: 354d) are analyzed in this work (Table 1). Since the model including all five Mg$^{2+}$ ions present in the crystal structure was found to be partially unstable, simulations 4MgA and 4MgB take into account only a subset of four Mg$^{2+}$ ions obtained by splitting into two the binuclear metal cluster formed by the ions 4A and 4B (the simulation 4MgA excludes ion 4B and the simulation 4MgB excludes ion 4A). In simulation NoMg the starting model is devoid of Mg$^{2+}$ ions and the concentration of added KCl is close to 0.2 M. Simulation NoMg1M is similarly devoid of Mg$^{2+}$ ions but the concentration of added KCl was raised to 1.0 M. Some features of the 4MgM1B and NoMg simulations have been described elsewhere.

The starting duplexes and associated Mg$^{2+}$ ions were placed in a box containing SPC/E water molecules in the amount necessary to ensure a 12 Å solvation shell around them. An appropriate number of K$^+$ and Cl$^-$ ions is added in order to obtain a concentration of KCl close to 0.2 M and 1.0 M. In order not to bias the positioning of the monovalent ions, they were placed around the solute on the basis of the electrostatic potential of the solvated system in the way that no ion was closer than 8 Å to any solute atom and 4 Å to any other ion. Thus, no monovalent ion is initially present in the deep groove.

The simulations were run at constant temperature (298 K) and pressure (1 atm = 101,325 Pa) by using the AMBER 6.0 simulation package. The calculations employed the all atom force-field described by Cornell et al. The van der Waals parameters for the K$^+$ and Cl$^-$ ions, calibrated for the SPC/E water model, were extracted from the following studies. The particle mesh Ewald (PME) summation method was used for the treatment of long-range electrostatic interactions. The chosen charge grid spacing is close to 1 Å and a cubic interpolation scheme was used. A cut-off of 9 Å for the van der Waals interactions and the Berendsen coupling scheme with a time constant of 0.4 ps were used. The standard PME parameters defined by AMBER led to an averaged Ewald error of 0.00008. Each trajectory was run with a 2 fs time step by using SHAKE bond constraints. The equilibration procedure which extends over 1.45 ns is identical with that reported elsewhere. The following 10 ns trajectories were subjected to conformational analysis using the AMBER package tools and our own procedures.

The ion pseudo-electron densities were calculated by using a procedure originally developed by Schneider & Berman and adapted by us. In short, all the ions that are located at less than 3.5 Å from any atom of a given base-pair were retained in order to form an “ion building block”. Building blocks for all pairs were subsequently rms-fitted on the average duplex conformation from the appropriate trajectory and assembled in order to give a “global ion building block” which was then Fourier transformed into a density map using the SFALL program of the CCP4 library. The 3.5 Å distance criterion was chosen because it matches the first minimum of the Ow...K$^+$ radial distribution function. Such a procedure minimizes the blurring effects of the densities at the end of the duplex with respect to those calculated for the central pairs, since these densities are first calculated at a local level and then assembled in order to reconstruct the entire duplex.

K$^+$-binding sites were placed in the calculated densities using the PEAKMAX program of the CCP4 library. Only the highest peaks (above a density level of 0.7 e/Å$^3$) were retained and considered as ion-binding sites (see Figure 5). If two peaks were found within a distance of 1.5 Å, they were averaged to a new position using a weight procedure in which contributions of both peaks were proportional to the third power of the peak height. An ion is considered as occupying an ion-binding region if it is found within a radius of 2.5 Å from any of the peaks associated with this region. The O8 program was used to visualize the “pseudo-electron” densities and the MDdraw program to visualize the MD trajectories.

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† [http://www.dl.ac.uk/CCP/CCP4](http://www.dl.ac.uk/CCP/CCP4)
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