Effects of magnesium ions on two EMCV IRES key activity fragments

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Abstract Using NMR magnetization transfer from water and ammonia-catalyzed exchange of the imino protons, changes have been monitored in base-pair kinetics induced by Mg^{2+} in two key activity fragments $r(CACCUGGCGACAGGUG)$ and $r(GGCCAAAAGCC)$ of the encephalomyocarditis virus internal ribosome entry site. For $r(CACCUGGCGACAGGUG)$, the addition of Mg^{2+} reveals two types of base-pairs: $r(U^{545}.A)$ and $r(G^{546}.C)$, in the first category, have lifetimes only slightly higher in the presence of Mg^{2+} , whereas their dissociation constants are substantially reduced. This behavior has been termed proximal. The base-pairs $r(G^{553}.C)$ and $r(G^{554}.C)$, in the second category, have lifetimes substantially higher in the presence of Mg^{2+} , whereas their dissociation constants remain almost constant. This behavior has been termed distal. Mg^{2+} has a specific effect on $r(CACCUGGCGACAGGUG)$, the magnitude of which is progressively modulated from the proximal region of the 16-mer towards its distal region. For $r(GGCCAAAAGCC)$, an intermediate behavior is found for base-pairs $r(G^{565}.C)$ and $r(G^{572}.C)$. Their lifetimes are slightly higher in the presence of Mg^{2+} and their dissociation constants are significantly lower, a behavior resembling that of the 16-mer proximal region. These results indicate that Mg^{2+} diffusively moves around $r(GGCCAAAAGCC)$.

Keywords Base-pair kinetics · EMCV IRES · GNRA tetraloop · Imino proton exchange · Mg^{2+} · NMR · RAAA hairpin motif

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Fig. 1 Schematic diagram showing the EMCV IRES hammerhead secondary structure containing the 16-mer (the proximal part in *red* and the distal in *blue*) and the 11-mer (in *mauve*) hairpins. The four residues spanning 5'-[G⁵⁴⁷–A⁵⁵⁰]-3' form the GCGA tetraloop. The four residues spanning 5'-[A⁵⁶⁸–A⁵⁷¹]-3' constitute the AAAA tetranucleotide motif

1 Introduction

We have recently established that the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) key activity RNA fragment r(CACCUGGCGACAGGUG), containing the GCGA tetraloop, adopts two topologies [1], a palindromic duplex with two conformations and a monomeric hairpin; the duplex form is destabilized by addition of Mg^{2+} and by temperature increase, the hairpin lifetime is around 6 months. In contrast, we have also shown that the EMCV IRES key activity RNA fragment r(GGCCAAAAGCC) containing the AAAA submotif adopts only a hairpin topology [1]. The present paper is an extension of our earlier article [1]. It completes the experimental study of Mg^{2+} effects on the two RNA fragments r(CACCUGGCGACAGGUG) and r(GGCCAAAAGCC) (see Fig. 1). Here, NMR has been used to determine the exchange properties of their base-pair imino protons catalyzed by ammonia under different $MgCl_2$ conditions. In terms of base-pair kinetic parameters, we distinguished two categories of base-pair, which we designated as the “proximal” type and the “distal” type. The proximal base-pairs are positioned closer to the GCGA tetraloop, and the distal base-pairs are farther away along the stem. The most conspicuous difference between both types is that at 15°C, the proximal base-pairs have lifetimes of the order of a few milliseconds whereas the distal base-pairs have lifetimes of the order of several tens of milliseconds.

2 Material and methods

Imino proton exchange theory, oligonucleotide synthesis, and the NMR methods used have already been described [1].

2.1 Sample preparation

Under all experimental conditions used in this work, both fragments r(CACCUGGCGACAGGUG) and r(GGCCAAAAGCC) adopted a hairpin topology at pH 8.9 and are henceforward referred to as the 16-mer and the 11-mer. To reach this situation, both samples were heated to 90°C for 3 min, rapidly cooled in an ice-water bath, and subsequently kept at 4°C. The hairpin/duplex interconversion rates are extremely low under such conditions [1]. The addition of Mg^{2+} was carried out by adding microliter volumes from $MgCl_2$ stock

solutions, such that the stoichiometries of Mg^{2+} to RNA were in a ratio of 1, 2, or 3. The Mg^{2+} concentrations are henceforth designated as 1, 2, or 3 eq of Mg^{2+} . The RNA fragment concentrations were 0.2 mM; the Mg^{2+} concentrations were, therefore, 0.2 mM (1 eq), 0.4 mM (2 eq), and 0.6 mM (3 eq), respectively.

2.2 UV absorbance melting (T_m) experiments

UV melting curves were collected over a temperature range of 5–90°C on a Shimadzu 1700 UV/VIS dual beam spectrophotometer with a heating rate of 1.0°C/min, measuring absorbance at both 260 and 280 nm. Alternative heating rates (for instance 0.5°C/min) or cooling rather than heating gave essentially identical curves. The stoichiometries of Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Cu^{2+} , and Zn^{2+} to RNA were at a ratio of 3:1. The RNA fragment concentrations were always 0.05 mM, i.e., the cation concentrations were 0.15 mM.

3 Results

3.1 Mg^{2+} and Ca^{2+} effects on the melting temperatures (T_m) of the 16-mer

Melting temperatures, T_m , of the 16-mer hairpin at pH 8.9 were measured with and without Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Cu^{2+} , and Zn^{2+} (Table 1). In the absence of added cations, the 16-mer hairpin at pH 8.9 unfolds in a single transition with a T_m of 71°C. With Mg^{2+} its T_m is 84°C and with Ca^{2+} equals 86°C, i.e., its stability is enhanced upon addition of Mg^{2+} or Ca^{2+} . In contrast, with added Mn^{2+} or Zn^{2+} the 16-mer T_m is 69°C; upon addition of Co^{2+} and Cu^{2+} it is 72°C and 73°C, respectively. Addition of Mn^{2+} , Zn^{2+} , Co^{2+} , or Cu^{2+} , therefore, has little influence on the 16-mer T_m . The greater stability of the 16-mer hairpin upon Mg^{2+} addition may result from a Mg^{2+} -induced local reorganization due to interactions of the cation with the GCGA tetraloop. The 16-mer hairpin $r(\text{G}^{546})$ site adjacent to the GCGA tetraloop (Fig. 1) constitutes a good candidate for binding a Mg^{2+} ion [2], which may compensate the adverse conformational entropy change of the GNRA reversed backbone necessary to allow formation of the two antiparallel strands of the stem [3]. In contrast, the 11-mer melting transition is at much lower temperatures since only two or three hydrogen bonds are disrupted by the input of thermal energy (Fig. 1, in mauve). The 11-mer T_m is approximately 40°C in the absence of any added cations and it increases to 43°C upon addition of Mg^{2+} .

3.2 Mg^{2+} -parameterized ammonia-catalyzed imino proton exchange

The 16-mer hairpin base-pairs $r(\text{U}^{545} \cdot \text{A})$, $r(\text{G}^{546} \cdot \text{C})$, $r(\text{G}^{553} \cdot \text{C})$, and $r(\text{G}^{554} \cdot \text{C})$, and the 11-mer hairpin base-pairs $r(\text{G}^{565} \cdot \text{C})$ and $r(\text{G}^{572} \cdot \text{C})$ were utilized as probes of the Mg^{2+} local effects on the kinetics of base-pair opening-closing. The imino proton of G^{549} is not

Table 1 Melting temperature variations of the 16-mer hairpin with and without 6 successive divalent cations

Cations	–	Mg^{2+}	Ca^{2+}	Mn^{2+}	Co^{2+}	Cu^{2+}	Zn^{2+}
T_m (°C)	71 ± 2	84 ± 2	86 ± 2	69 ± 2	72 ± 2	73 ± 2	69 ± 2
ΔT_m (°C)	0 ± 4	13 ± 4	15 ± 4	-2 ± 4	1 ± 4	2 ± 4	-2 ± 4

The cation concentrations are 0.15 mM

Fig. 2 Ammonia contributions to the exchange times of the $r(G^{553}\cdot C)$ and $r(U^{545}\cdot A)$ imino protons of the 16-mer hairpin, both with and without Mg^{2+} at 5°C and 15°C. The slopes of the plots indicate a slight decrease of the $r(G^{553}\cdot C)$ dissociation constant and a change of the $r(U^{545}\cdot A)$ dissociation constant, whereas extrapolations to infinite ammonia concentration show an increase of the $r(G^{553}\cdot C)$ lifetime and a slight change of the $r(U^{545}\cdot A)$ lifetime upon addition of Mg^{2+}

observed by NMR since it belongs to a base which is not paired and, therefore, exchanges rapidly with solvent on the chemical shift timescale, even without addition of ammonia. The imino proton of G^{547} in the 16-mer also exchanges rapidly with solvent as soon as a few microliters of ammonia are added, since it is partially involved in a non-canonical $r(G^{547}\cdot A)$ base-pair. Additionally, the imino protons of the 16-mer base-pairs $r(U^{555}\cdot A)$ and $r(G^{556}\cdot C)$, and the 11-mer base-pair $r(G^{564}\cdot C)$ behave similarly, because they are at the stem extremities and are, therefore, subject to end-fraying. The ammonia contribution, τ_{ex, NH_3} , to imino proton exchange in the 16-mer displayed in Fig. 2 is typical of the measurements performed in the presence or absence of Mg^{2+} . Comparison of base-pair kinetics in the two RNA fragments with and without Mg^{2+} at 15°C is given in Table 2. The variations of base-pair lifetimes, τ_0 , and base-pair dissociation constants, K_{diss} , with temperature give, respectively, the opening activation energies, E_a , and the dissociation enthalpy differences, ΔH . The product $\tau_0 K_{diss}$ equals the lifetime of the open pair, τ_{open} . The kinetics of the proximal 16-mer base-pairs and the distal 16-mer base-pairs are analyzed separately, with and without Mg^{2+} ; the 11-mer base-pair case is then discussed.

1. Kinetics of the proximal 16-mer base-pairs $r(U^{545}\cdot A)$ and $r(G^{546}\cdot C)$, with and without Mg^{2+}

The base-pair $r(U^{545}\cdot A)$ is a typical proximal 16-mer base-pair. Without Mg^{2+} , its lifetime, obtained by extrapolation of the NH_3 exchange contribution to infinite ammonia concentration, is 1.8 ms and its dissociation constant is 140×10^{-6} at 15°C. With 1 eq of Mg^{2+} , its lifetime remains almost constant and equals 1.4 ms whereas its dissociation constant increases to 350×10^{-6} ; with 3 eq of Mg^{2+} , its lifetime increases to 3.2 ms and its dissociation constant is then 600×10^{-6} . The base-pair $r(U^{545}\cdot A)$ thus has a relatively short τ_0 and a relatively large K_{diss} . Upon Mg^{2+} addition, its lifetime varies little, whereas its dissociation constant exhibits a relatively large increase. At 5°C, without Mg^{2+} , its lifetime is 1.1 ms and its dissociation constant is 34×10^{-6} . With 1 eq of Mg^{2+} , its lifetime becomes

Table 2 Lifetimes, dissociation constants and open pair lifetimes in base-pairs of the 16-mer and of the 11-mer hairpins at 15°C

		[Mg ²⁺]	τ_0 (ms)	$K_{\text{diss}} \times 10^6$	τ_{open} (ns)	ΔH (kJ/mol)	E_a (kJ/mol)
16-mer	<u>U</u> ⁵⁴⁵	0 eq	1.8	140	240	81	7.2
		1 eq	1.4	350	490	120	53
		3 eq	3.2	600	1900	93	24
	<u>G</u> ⁵⁴⁶	0 eq	4.8	21	101	100	32
		1 eq	6.5	7.6	49	120	96
		3 eq	11	15	160	110	130
	G ⁵⁵³	0 eq	22	1.6	35	7.8	2.2
		1 eq	41	4.0	160	19	29
		3 eq	43	2.2	95	46	30
	G ⁵⁵⁴	0 eq	18	1.6	29	2.6	23
		1 eq	25	1.9	47	16	30
		3 eq	51	2.0	99	24	74
11-mer	G ⁵⁶⁵	0 eq	18	39	700	—	46
		1 eq	21	8.8	180	—	35
		3 eq	21	6.2	130	—	24
	G ⁵⁷²	0 eq	11	4.9	54	—	—
		1 eq	13	9.5	120	—	—
		3 eq	11	11	120	—	—

The bases belonging to the proximal base-pairs of the 16-mer hairpin are underlined. The uncertainties on base-pair lifetimes are estimated as $\pm 30\%$. The uncertainties on dissociation constants are about $\pm 20\%$

— Non measurable because of fast exchange with water on the chemical shift scale

0.7 ms and its dissociation constant increases to 86×10^{-6} ; with 3 eq of Mg²⁺, its lifetime equals 2.2 ms and its dissociation constant is then 101×10^{-6} . The behavior of r(U⁵⁴⁶.C) is similar. The characteristics of the proximal base-pairs upon addition of Mg²⁺ can be summarized as follows: a small τ_0 that changes ($\Delta\tau_0$) little and a large K_{diss} that changes (ΔK_{diss}) substantially.

2. Kinetics of the distal 16-mer base-pairs r(G⁵⁵³.C) and r(G⁵⁵⁴.C), with and without Mg²⁺

The base-pair r(G⁵⁵³.C) is a typical distal 16-mer base-pair. Without Mg²⁺ its lifetime is 22 ms and its dissociation constant is 1.6×10^{-6} at 15°C. With 1 eq of Mg²⁺, its lifetime increases to 41 ms and its dissociation constant increases to 4.0×10^{-6} ; with 3 eq of Mg²⁺, its lifetime equals 43 ms and its dissociation constant is then 2.2×10^{-6} . The r(G⁵⁵³.C) base-pair thus has a relatively long τ_0 and small K_{diss} . Upon Mg²⁺ addition, its lifetime exhibits a relatively large increase, whereas its dissociation constant varies only slightly. At 5°C, without Mg²⁺, the lifetime of r(G⁵⁵³.C) is 27 ms and its dissociation constant is 0.9×10^{-6} , and with 1 eq of Mg²⁺, its lifetime increases to 90 ms and its dissociation constant increases to 1.4×10^{-6} . With 3 eq of Mg²⁺, its lifetime increases further to 100 ms and its dissociation constant is then 1.2×10^{-6} . The behavior of r(U⁵⁵⁴.C) is similar. The hallmarks of these distal base-pairs are thus: upon addition of Mg²⁺, the large τ_0 becomes even larger and its small K_{diss} does not change much.

3. Kinetics of the 11-mer base-pairs r(G⁵⁶⁵.C) and r(G⁵⁷².C), with and without Mg²⁺

In the absence of Mg²⁺, the 11-mer r(G⁵⁶⁵.C) and r(G⁵⁷².C) base-pair lifetimes are equal to 18 and 11 ms, respectively. With 3 eq of Mg²⁺, they become 21 and 11 ms, respectively. The dissociation constant of r(G⁵⁶⁵.C) is quite large without Mg²⁺, having a value of 39×10^{-6} .

With 3 eq of Mg^{2+} added, however, it increases by a factor of 6 to 6.2×10^{-6} . The lifetime of the $r(\text{G}^{565} \cdot \text{C})$ open state is of the order of several hundred nanoseconds and decreases to ~ 130 ns in the presence of 3 eq of Mg^{2+} . In contrast, the $r(\text{G}^{572} \cdot \text{C})$ dissociation constant is relatively low without Mg^{2+} , having a value of 4.9×10^{-6} . With 3 eq of Mg^{2+} , it increases by a factor of 2 to 11×10^{-6} . The lifetime of the $r(\text{G}^{572} \cdot \text{C})$ open state is of the order of 50 ns, increasing to approximately 120 ns in the presence of 3 eq of Mg^{2+} . The hallmarks of the two 11-mer base-pairs upon addition of Mg^{2+} are thus: a moderate or large τ_0 but a small change, $\Delta\tau_0$; a moderate or large K_{diss} and a moderate or large change, ΔK_{diss} .

4 Discussion

The base-pair kinetic analysis leads to a mapping of Mg^{2+} -mediated effects and to a characterization of the propagation of these effects along and across the 16-mer and 11-mer hairpin stems. The 16-mer hairpin appears to be divided into two parts: a proximal part composed of $r(\text{U}^{545} \cdot \text{A})$ and of $r(\text{G}^{546} \cdot \text{C})$ and a distal part composed of $r(\text{G}^{553} \cdot \text{C})$ and $r(\text{G}^{554} \cdot \text{C})$. Mg^{2+} preferential effects for the 16-mer hairpin may explain the variations of its base-pair opening-closing kinetics which strongly suggest a structural effect of the cation. This observation supports the hypothesis of Mg^{2+} /GCGA tetraloop binding, which may provide a nucleation site to ensure proper folding of the larger RNA [4–6]. The K_{diss} of $r(\text{U}^{545} \cdot \text{A})$ and $r(\text{G}^{546} \cdot \text{C})$ in the 16-mer varies considerably on addition of Mg^{2+} , whereas the K_{diss} of $r(\text{G}^{553} \cdot \text{C})$ and $r(\text{G}^{554} \cdot \text{C})$ remains largely unaltered, suggesting that Mg^{2+} serves to enhance the 16-mer stability [3]. Whatever the precise position of the bound Mg^{2+} , the proximal 16-mer base-pair $r(\text{G}^{546} \cdot \text{C})$ has its imino proton less exposed to the solvent in the presence of 3 eq of Mg^{2+} , whereas the distal part of the 16-mer has its base-pairs more accessible to the solvent. The Mg^{2+} effects also seem to propagate from the proximal to the distal part of the 16-mer hairpin (Table 2). Along the path from the end of the 16-mer GCGA tetraloop to the beginning of the 16-mer stem (Fig. 1), the magnitude of the Mg^{2+} effects follows a gradient of intensity. On addition of 3 eq of Mg^{2+} , the base-pair lifetimes increase from $r(\text{U}^{545} \cdot \text{A})$ ($\times 1.8$), to $r(\text{G}^{554} \cdot \text{C})$ ($\times 2.8$) and, correspondingly, the decrease of dissociation constants goes from $r(\text{U}^{545} \cdot \text{A})$ ($\times 4.3$) to $r(\text{G}^{554} \cdot \text{C})$ ($\times 1.2$). The 11-mer base-pair kinetics suggests a uniform distribution of the Mg^{2+} cations around the hairpin: $r(\text{G}^{565} \cdot \text{C})$ and $r(\text{G}^{572} \cdot \text{C})$ have their K_{diss} reduced on adding Mg^{2+} and their lifetimes are comparable to those in the absence of Mg^{2+} . They exhibit a behavior resembling that termed proximal in the 16-mer. A plausible explanation might be that purines close to a loop partake in a specific local conformation in the presence of Mg^{2+} , which involves the base-pairs in their vicinity [2]. Such arguments connected to the unknown tertiary structure might also explain the energy differences observed for the base-pairs immediately adjacent to the loop and those in the stem (Table 2).

5 Conclusion

We distinguish three categories of base-pair upon Mg^{2+} addition. These are summarized in Fig. 3 which shows the 16-mer and 11-mer base-pair properties averaged over temperature (5°C , 15°C , and 25°C) and magnesium concentration. The base-pair K_{diss} values are represented by empty squares and the base-pair lifetimes, τ_0 , by circles. The 11-mer base-pairs are in mauve; they seem to adopt an intermediate regime as compared to the 16-mer

Fig. 3 Classification of Mg²⁺ effects on the 16-mer and 11-mer hairpins, leading to the distinction between the 16-mer proximal and distal behaviors and the existence of an intermediate situation for the 11-mer. The base-pair dissociation constants (*squares*) and the base-pair lifetimes (*circles*) are averages, enabling an overview of the distribution of base-pair kinetics and changes in their properties. The bases surrounded by white halos are those whose measured properties are given in Table 2

proximal and distal base-pairs: upon Mg²⁺ addition, they show relatively moderate $\Delta\tau_0$ and ΔK_{diss} . The constant value of τ_0 for r(G⁵⁷².C) in the 11-mer indicates that Mg²⁺ moves diffusively without altering any specific sites of the 11-mer hairpin. The 16-mer proximal base-pairs are in red: upon Mg²⁺ addition, a small $\Delta\tau_0$ and relatively moderate ΔK_{diss} are observed. The 16-mer distal base-pairs are in blue: upon Mg²⁺ addition, a large $\Delta\tau_0$ and small ΔK_{diss} occur. The variations of τ_0 for r(U⁵⁴⁵.A), r(G⁵⁴⁶.C), r(G⁵⁵³.C), and r(G⁵⁵⁴.C) in the 16-mer, with and without Mg²⁺, show that this alteration of the exchange kinetics is related to local effects of Mg²⁺. The 16-mer hairpin r(G⁵⁴⁶) is part of the closing base-pair between the loop and the stem; it constitutes a strong candidate for binding a Mg²⁺ ion [2]. Despite the presence of other potential binding sites, Mg²⁺ seems to have a preferential effect on a specific region of the 16-mer hairpin, involving at least r(G⁵⁵³.C), r(G⁵⁵⁴.C), r(U⁵⁴⁵.A), and r(G⁵⁴⁶.C), and it may be classified as an ion bound specifically. The proximal base-pair r(G⁵⁴⁶.C) appears less dissociated upon addition of Mg²⁺, and this is where the Mg²⁺ ion might be bound [2, 4–6], making its immediate molecular vicinity more rigid. An opposite tendency seems to prevail from the proximal base-pair r(U⁵⁴⁵.A) towards the distal part of the 16-mer. The distal region seems more flexible in the presence of Mg²⁺. The stability situation appears to become gradually modulated from the proximal base-pair r(G⁵⁴⁵.C) to the distal base-pair r(G⁵⁵⁴.C), along and across the 16-mer hairpin stem. To judge whether or not these variations on the addition of Mg²⁺ are necessary contributions to the folding of the hammerhead region requires further investigations on the whole Domain 3 of the EMCV IRES.

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