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The role of salt concentration and magnesium binding in HIV-1 subtype-A and subtype-B kissing loop monomer structures

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Abstract

The subtype-B monomers of the human immunodeficiency virus type-1 (HIV-1) have experimentally been shown to dimerize at high salt concentration or in the presence of magnesium, while the dimerization of the subtype-A monomers requires magnesium binding at the G₂₇₃ or G₂₇₄ phosphate groups regardless of salt concentration. We used explicit solvent molecular dynamics (MD) simulations to investigate the conformational changes in subtype-A and -B monomers in different salt concentrations, and we found that our MD simulation results are consistent with those of experiments. At low salt concentration, hairpin loop structures of both subtypes were deformed and bases in the hairpin loop were turned inside. At high salt concentrations, the subtype-B monomer maintained the hairpin loop shape and most bases in the hairpin loop pointed out, while the subtype-A monomer showed a severe deformation. We also found that the flanking bases in the subtype-B stabilize the hairpin loop, while the flanking base G₂₇₃ in the subtype-A caused a significant deformation. However, a bound magnesium ion at the G₂₇₃ or G₂₇₄ phosphate groups controlled the behavior of the G₂₇₃ base and prevented the subtype-A monomer from deformation. We also applied restraints to both subtypes to examine the role of high salt concentration or magnesium binding. While restraints were applied, both subtypes at 0 M salt concentration maintained their shapes. However, when restraints were removed, they deformed significantly. Therefore, we suggest that the dimerization of both subtypes requires the proper conformation of the monomers which is induced by the appropriate salt strength and magnesium ion binding.

Keywords

HIV-1 dimerization; HIV-1 subtype-A dimerization; HIV-1 subtype-B dimerization; Ion concentration; Magnesium binding; RNA molecular dynamics simulations

Introduction

The dimerization initiation site (DIS) of human immunodeficiency virus type 1 (HIV-1) has a hairpin loop structure, which contains six nucleotides with a self-complementary sequence (SCS) and two and one flanking purines at the 5' and 3' ends of the loop, respectively. The kissing loop formation takes place by intermolecular Watson–Crick interactions between six

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nucleotides in the SCS of two copies of the hairpin loop structures (Laughrea & Jette, 1994; Muriaux, Girard, Bonnet-Mathoniere, & Paoletti, 1995; Skripkin, Paillart, Marquet, Ehresmann, & Ehresmann, 1994). At higher temperature (55 °C) or in the presence of nucleocapsid protein, the kissing loop interaction is changed into a more stable conformation, an extended duplex structure (Laughrea & Jette, 1996; Muriaux, Fosse, & Paoletti, 1996; Muriaux, Rocquigny, Roques, & Paoletti, 1996). However, mutations in the SCS decrease the replication rate and therefore reduce the infectivity of HIV-1 virus (Berkhout & van Wamel, 1996; Haddrick, Lear, Cann, & Heaphy, 1996; Paillart et al., 1996). The DIS loop sequence of subtype-A is A₂₇₂GGUGCACA₂₈₀ and that of subtype-B is A₂₇₂AGCGCGCA₂₈₀ (Figure 1(a)). In the X-ray structure (Ennifar & Dumas, 2006), the flanking purines A₂₇₂, G₂₇₃ in subtype-A (PDB code: 1XPF) and A₂₇₂, A₂₇₃ in subtype-B (PDB code: 1XPE) are pointing out, while A₂₇₂ in the NMR structure of subtype-B (PDB code: 2X4F) (Kieken, Paquet, Brule, Paoletti, & Lancelot, 2006) is partly pointing in.

The dimerization of subtypes-A and -B differs in their dependence on ion concentration and ion types. Experimentally, it is observed that subtype-B can dimerize at high salt concentrations (.3 M) without magnesium, low salt concentrations (.04 M) with magnesium (.1 mM), and high salt concentrations (.3 M) with magnesium (5 mM). At the low salt concentration without magnesium, the subtype-B is found to exist as a monomer. On the other hand, subtype-A dimerizes only under high salt concentration with high concentration of magnesium ions (Lodmell et al., 1998). Another difference between subtype-A and -B is that subtype-A dimerization requires magnesium ion binding at the G₂₇₃ or G₂₇₄ phosphate groups, while subtype-B does not require magnesium binding (Jossinet et al., 1999). Brownian simulations also show that these phosphate groups in the subtype-A have higher probability of magnesium binding than the corresponding regions of subtype-B (Jossinet et al., 1999). It has been unknown why subtype-A dimerization requires magnesium binding at the G₂₇₃ or G₂₇₄ phosphate groups, while subtype-B dimerization does not.

Lodmell, Ehresmann, Ehresmann, and Marquet (2000) used SELEX to investigate sequence dependence and structural constraints on dimerization of the HIV-1 DIS by randomizing nucleotides in the DIS loop. It is shown by *in vitro* selection of dimerization-competent sequences that positions 272 and 280 have a strong tendency to be an adenine. Since a non-Watson-Crick interaction exists between positions 272 and 280 (Clever, Wong, & Parslow, 1996), this result may imply a canonical base pairing between positions 272 and 280 is disfavored for dimer formation. Although position 273 shows a tendency for purines, it strongly depends on the bases at both positions 276 and 277. Lorenz et al. studied the stability of kissing loop interactions with constant core sequences (5'-CCGACC-3') where hairpin loop structures have different types and number of flanking bases at the 5' and the 3' sides (Lorenz, Piganeau, & Schroeder, 2006; Weixlbaumer, Werner, Flamm, Westhof, & Schroeder, 2004). In their UV melting experiments, HIV-1 DIS-like kissing loop sequences (5'-AACCGACCA-3' and 5'-AGCCGACCA-3') are found to be the most stable. On the other hand, the 5'-ACCGACCA-3' loop is less stable than the HIV-1 DIS-like kissing loop, but is more stable than 5'-ACCGACCAA-3' or 5'-GCCGACCAA-3'. Therefore, the flanking bases at positions 271, 272, and 280 may have different roles in determining dimerization and the stability of kissing loop interactions under different salt concentrations. One possible explanation for the role of the flanking bases for dimerization is the formation

of non-Watson–Crick interactions between flanking purines. However, it was found that a non-Watson–Crick interaction between A₂₇₂ and A₂₈₀ is not required for dimerization (Jossinet et al., 1999) and the exact role of the flanking bases for dimerization of HIV-1 DIS is still an open question.

It is important to understand how different salt concentrations affect the dimerization of two monomers via kissing loop formation, not only for antiretroviral therapy, but also for developing RNA-based applications such as designing RNA nanoparticles. For example, one tectoRNA building block contains two hairpin loop structures (Chworos et al., 2004; Hansma, Oroudjev, Baudrey, & Jaeger, 2003; Jaeger, Westhof, & Leontis, 2001; Koyfman et al., 2005) and self-assembles into tectosquares by kissing loop interactions in the presence of magnesium. RNAIi and RNAIii kissing loops have a 120° corner angle and they self-assemble into a RNA hexagonal ring in the presence of magnesium ions (Grabow et al., 2011; Paliy, Melnik, & Shapiro, 2009, 2010; Yingling & Shapiro, 2007). Understanding the role of ion concentration and the effect of magnesium binding would aid in the design of additional RNA nanoparticles.

Molecular dynamics (MD) simulations of the full kissing loop complexes of subtype-A, -B, and -F have already been studied using X-ray and NMR structures to investigate the behavior of the flanking bases with only neutralizing ions (K⁺, Na⁺, and Mg²⁺) (Reblova et al., 2007). They found that the dimer structures were not only stable, they also found that the flanking bases prefer to bulge out and form a closed conformation, in which the four flanking purine bases stack together. However, MD simulations of the HIV-1 monomers have not been performed. The monomer structures of the subtype-A and -B in different salt conditions are not available in X-ray or NMR structures. The only available HIV-1 DIS monomer structure is a mutated subtype-B NMR structure, where G₂₇₆ in the SCS is mutated to A₂₇₆ to prevent homodimerization (PDB code: 1JTJ) (Kieken et al., 2002). Therefore, we separated the monomers from the full kissing loop complexes and used 100 ns long explicit solvent MD simulations to investigate the dynamics of subtype-A and -B monomers under different salt conditions. We did not consider using implicit MD simulations since explicit MD simulations can provide a higher degree of accuracy and in addition explicit MD simulations are required to obtain information on the interactions between the monomers, water, and ions. Despite the fact that the monomer structures lost their dimerization partners, subtype-B monomers maintained their shapes in high salt concentrations or in high salt concentration with magnesium ions. On the other hand, the subtype-A monomer was significantly deformed in high salt concentration. We also found that the flanking bases in subtype-B stabilized the hairpin loop conformations while the flanking base G₂₇₃ in subtype-A played a major role of causing significant deformations regardless of the salt concentration. However, a magnesium ion bound at the G₂₇₃ or G₂₇₄ phosphate group prevented deformation and contributed to the maintenance of the appropriate shape for kissing loop formation.

Materials and methods

We separated the monomers from the full kissing loop complexes and examined them under different salt concentrations. In the hairpin loop structure, we denoted 274, 275, and 276 as

the upper hairpin loop bases and 277, 278, and 279 as the lower hairpin loop bases. For the MD simulations of neutralized monomers, all ions in the initial monomer structures (2F4X, 1XPE, and 1XPF) were removed and 22 Na⁺ ions were placed to neutralize the RNA backbone phosphate groups. We regard this neutralized condition as 0 M salt concentration. For the low and high salt concentration MD simulations, .05 and .35 M NaCl were added to the neutralized monomers. For the MD simulations with magnesium ions, the two magnesium ions (Mg(1) and Mg(2)) in 1XPE and the three magnesium ions (Mg(1), Mg(2), and Mg(3)) in 1XPF were preserved and 18 Na⁺ and 16 Na⁺ ions were placed to neutralize 1XPE and 1XPF, respectively. To increase the salt concentration, .35 M NaCl and .07 M MgCl₂ were added. Since the initial location of Mg(1) and Mg(2) in the subtype-A are close to the G₂₇₄ and G₂₇₆ phosphate groups, respectively (3.7 and 2.3 Å, see Figure 1(b)), they bound to each phosphate group as soon as the MD simulations started. On the other hand, it has experimentally been shown that the G₂₇₃ and G₂₇₄ phosphate groups are important magnesium binding sites for dimerization. Therefore, we performed two additional 100 ns long MD simulations to study when a magnesium ion (Mg(1)) is bound to each of phosphate group, Mg (1)-G₂₇₃ and Mg(1)-G₂₇₄, respectively. Since it takes microsecond time scales to simulate dehydration of magnesium ions and the formation of Mg²⁺ O binding (Hashem & Auffinger, 2009; Ohtaki, 2001; Ohtaki & Radnai, 1993), we relocated the initial positions of magnesium ion to induce appropriate magnesium binding. The MD simulation of Mg(1)-G₂₇₄ was prepared by increasing the initial distance between Mg(2) and the G₂₇₆ phosphate group from 2.3 to 5.4 Å to avoid Mg(2) binding. This way, only Mg(1) can bind to the G₂₇₄ as MD simulation starts. The MD simulation of Mg(1)-G₂₇₃ was prepared by decreasing the initial distance between Mg(1) and the G₂₇₃ phosphate group from 5.9 to 2.0 Å, while the initial distance of Mg(2) from the G₂₇₆ phosphate group increased to 5.4 Å. Therefore, Mg(1) can bind to the G₂₇₃ phosphate group at the beginning of the MD simulation. All initial structures and their test groups consisting of monomers (2F4X, 1XPE, and 1XPF) with different salt concentrations are listed in the supplementary material (Table S1).

A TIP3P water (Jorgensen, Chandrasekhar, Madura, & Klein, 1983) box was added so that there was a 18 Å distance from each side of the RNA. MD simulations were performed using the Amber 10 MD simulation package (Case et al., 2008) with the ff99 Cornell force field (Cheatham III, Cieplak, & Kollman, 1999). The ff99 force field was also used in previous MD simulations of kissing loop complexes under various salt conditions. All maintained a high degree of stability (Kasprzak, Bindewald, Kim, Jaeger, & Shapiro, 2011; Kim, Marquez, Barchi, & Shapiro, 2011; Reblova et al., 2007; Reblova, Spackova, Sponer, Koca, & Sponer, 2003). For the ions, the following parameters were used: Na⁺ radius 1.868 Å and well depth .00277 kcal/mol; Mg²⁺ radius .7926 Å and well depth .8947 kcal/mol; and Cl⁻ radius 1.948 Å and well depth .265 kcal/mol. The particle mesh Ewald summation (PME) (Essmann et al., 1995) was used to calculate the electrostatic interactions. The minimization of the system was done using harmonic constraints on the RNA. After minimization, the system was heated to 300 K while constraining the solute with a 200 kcal/(molÅ) harmonic constraint. Constraints were slowly released as the system equilibrated. In order to remove the fastest hydrogen vibrations and to allow longer simulation time steps, SHAKE (Ryckaert, Ciccotti, & Berendsen, 1977) was applied to all hydrogen atoms. A constant temperature of 300 K was maintained using a weak-coupling algorithm (Berendsen,

Postma, Gunsteren, DiNola, & Haak, 1984) while the pressure was maintained at 1.0 Pa. Production simulations for low and high salt concentrations were performed for 100 ns with a 2 fs time step. Since monomers in the 0 M salt concentration were significantly deformed within 10 ns, production simulations for the 0 M salt concentration were performed for 50 ns. Average monomer structures in low and high salt concentrations including the presence of magnesium were obtained from the 50–100 ns MD simulation trajectory, while those of the 0 M salt concentration were obtained from the 20–50 ns MD simulation trajectory. Although, the average structure may not exist in the MD trajectory, it is precise enough to represent the shape of structure for the corresponding MD trajectory. For example, the minimum and the average root mean square deviation (RMSD) between the average structure of 2F4X and the corresponding trajectories in the high salt condition is less than .7 and 1.8 Å, respectively.

We determined the ability of each monomer to form kissing loop interactions by measuring the similarity between the MD simulation trajectories and the corresponding initial structures. The similarity was measured with RMSD of the complete monomer (C₂₆₅–G₂₈₇), its stem (C₂₆₅–G₂₇₁ and C₂₈₁–G₂₈₇), and its hairpin loop (A₂₇₂–A₂₈₀) regions with respect to the corresponding initial structures by using the backbone atoms (P, O5', C5', C4', C3', and O3'). All RMSD values are listed in the supplementary material (Table S2). In addition, we computed the global distance test total score (GDT_TS) (Zelma, 2003) between the initial and the average structures. These values are listed in the supplementary material (Table S5). We also used the deformation profile matrix (DPM) (Parisien, Cruz, Westhof, & Major, 2009) to obtain detailed information about the deformations. In DPM, each nucleotide in the average structures was aligned to its corresponding nucleotide in the initial structure. For each alignment, the distance between nucleotides in the initial and the average structures was calculated.

The distance between N1(271) and P(277), which is relatively parallel to helical axis is denoted as the horizontal loop component while the distance between P(273) and P(280), which is perpendicular to the helical axis is denoted as the vertical loop component to describe the change of hairpin loop shape. (see Figure 1 (e)). A loop shape ratio was calculated by the ratio between vertical and horizontal loop components. The flattening of the initial hairpin loop curvature along G₂₇₁–G₂₇₆ was measured by the change in distance between P(267) and P(278) (see Figure 1(e)). These distances and loop shape ratios are listed in the supplementary material (Table S2). Hydrogen bond (HB) interactions in the hairpin loop region were monitored using a 3.5 Å (between heavy atoms) and 120° cutoff (between the acceptor, hydrogen, and donor atoms). Since the counterpart of the monomer was removed from the kissing loop complexes, donors and acceptors in the DIS hairpin loop region had no HB interaction at the beginning of the MD simulations. As the hairpin loop was deformed during the MD simulations, some of the bases in the DIS turned inside and established intrastrand HB interactions. The orientation of bases in DIS and HB occupancies are summarized in the supplementary material, Table S3 and Table S4. In addition, since the behavior of the subtype-B X-ray structure (1XPE) is similar to that of the NMR structure (2F4X), its average structures and DPM results are plotted in the supplementary material. The above analysis was performed for 50–100 ns time ranges and the same analysis was

applied to monomers in the 0 M salt concentration (neutralized system) for 20–50 ns time ranges.

Results and discussion

Subtype-B NMR monomer (2F4X) in different salt concentrations

At 0 M and low salt concentrations, due to the electrostatic repulsion between the hairpin loop and the stem, the initial sharp backbone curvature near the flanking bases was flattened and the hairpin loop shape was significantly deformed (see Figure 2(a) and (b)). The P(267)–P(278) distance, which measured the distance between the 5' side and hairpin loop backbone (see Figure 1(e)) increased from 12.4 to 31.3 ± 3.5 and 26.2 ± 3.8 Å for no and low salt concentrations, respectively (see Table S2). During the MD simulations, the horizontal loop component increased while the vertical loop component was decreased with respect to the corresponding initial structures. Therefore, the loop shape ratios (see Table S2) in both salt conditions were reduced from 1.7 to .8. The average RMSD values (see Figure 4 and Table S2) indicated that most of the deformations were caused by the hairpin loop distortion. As the hairpin loop structure was distorted, most bases in the SCS turned in and formed HB interactions (see Table S4). The flanking bases at 272 and 273 also pointed in and formed HB interactions with A₂₈₀.

As shown in Figure 2(c), the overall behavior of the monomer in the high salt concentration was significantly different from the previous cases. The sharp hairpin loop curvature along A₂₇₂–G₂₇₆ was retained for the entire simulation and the average distance between P(267) and P(278) was measured as 15.6 ± 2.8 Å. The longer vertical loop component (17.5 ± 1.7 Å) compared to the horizontal loop component (15.1 ± 1.1 Å) indicated that the hairpin loop shape was also well maintained. In addition, the four bases (G₂₇₄, C₂₇₅, C₂₇₇, and G₂₇₈) in the SCS turned out and were available for kissing loop formation. The hairpin loop RMSD showed the lowest value ($2.1 \pm .6$ Å) in the 2F4X test group and it contributed the lowest overall RMSD ($3.3 \pm .6$ Å). Therefore, these results indicated a strong correlation with experiments that subtype-B monomers dimerize at high salt concentration (Jossinet et al., 1999; Lodmell et al., 1998).

Subtype-B X-ray monomer (1XPE) in different salt concentrations

The MD simulations of the subtype-B X-ray structure showed similar patterns to those of the NMR structure. In 0 M and low salt concentrations, the P(267)–P(278) distance was increased and the loop shape ratio was reduced to less than .8. Four bases in the SCS were turned in and formed HB interactions in both 0 M and low salt concentrations. Due to the similarity between NMR and X-ray subtype-B monomers, the average structures of 1XPE are plotted in the supplementary material, Figure S1.

In the high salt concentration, the hairpin loop maintained its curvature and the P(267)–P(278) distance (see Figure S1(c) and Table S2). The loop shape ratio (1.14) also indicated a minimal deformation for the hairpin loop structure. The bases from G₂₇₄ to G₂₇₈ were pointed out and stacked on each other. The average RMSDs of the hairpin loop and the overall structure were $3.2 \pm .3$ and $3.8 \pm .6$ Å, respectively, which were as low as that of the

NMR structure in the high salt concentration. Therefore, high salt concentration could sustain the overall shape of the monomer after removing magnesium ions from its original X-ray structure.

When magnesium ions were present, the overall shape and the hairpin loop curvature also maintained the appropriate conformation. (Figure S1(d) and Table S2). Mg(1) at the center of the stem, which was from the original X-ray structure (see Figure 1(c)), stayed at the same location throughout the simulation and stabilized the stem. Several $\text{Mg}(\text{H}_2\text{O})_6$ complexes, which were added to the system to increase the magnesium concentration, were found between the stem ($\text{U}_{266}\text{--G}_{271}$) and the hairpin loop backbones ($\text{G}_{274}\text{--G}_{278}$). These nonbound magnesium ions also stabilized the monomer structure (Misra & Draper, 2001) by preventing flattening of the hairpin loop curvature. G_{274} and G_{278} pointed in and formed HB interactions, while four other bases in the SCS pointed out. Therefore, these MD simulation results showed strong correspondence with the previous experimental results that subtype-B monomers dimerize either in the presence or in the absence of magnesium ions (Jossinet et al., 1999; Lodmell et al., 1998).

Subtype-A X-ray monomer (1XPF) in different salt concentrations without magnesium ions

Compared to the subtype-B monomers, the subtype-A monomers showed more deformations. For example, both the stem and the hairpin loop were distorted at 0 M salt concentration (Figure 3(a)) and the hairpin loop region at the low salt concentration showed the largest RMSD value in the entire test group (see Table S2). For both 0 M and low salt concentrations, most of bases in the hairpin loop turned in and formed HB interactions. However, it was more interesting to find that the sub-type-A monomer had a large deformation in the high salt concentration (see Figure 3(c)). The stem's helix unwound and the stem RMSD increased to $7.3 \pm .6$ Å. The hairpin loop experienced a relatively small deformation in terms of a hairpin loop RMSD and a 1.3 loop shape ratio. However, a large deformation in the stem caused the P(267)–P(278) distance to increase to 29.0 ± 4.6 Å and the overall RMSD reached $9.0 \pm .5$ Å. Since experimental results show that the subtype-A monomers do not dimerize in high salt concentration (Jossinet et al., 1999; Lodmell et al., 1998), our results suggest that the appropriate shape of the monomer is important for HIV-1 kissing loop formation.

Subtype-A X-ray monomer (1XPF) with magnesium binding

When the MD simulation of the subtype-A monomer was performed with the presence of magnesium ions, the two magnesium ions (Mg(1) and Mg(2)) from the X-ray structure were bound to the phosphate groups in G_{274} and G_{276} , respectively. The third magnesium ion (Mg(3)) from the X-ray structure, which was initially located near the $\text{G}_{271}\text{--C}_{281}$ base pair, lost its position within 8 ns. Other magnesium ions, which were added to increase magnesium concentration, occupied the major groove and between the stem ($\text{U}_{266}\text{--G}_{271}$) and the hairpin loop backbone ($\text{G}_{274}\text{--G}_{278}$). At 35 ns, base pairing between G_{271} and C_{281} was broken and it induced a deformation of the hairpin loop, which caused the loop shape ratio to drop to .6. Compared to the RMSD values for the sub-type-B monomers in high salt concentrations, it had larger RMSD values.

It was experimentally found that the G₂₇₃ or G₂₇₄ phosphate groups are important magnesium binding sites for the dimerization of the subtype-A monomers (Jossinet et al., 1999). Previous Brownian simulations also indicate that these two phosphate groups are strong magnesium binding sites (Jossinet et al., 1999). Therefore, we performed two additional 100 ns long MD simulations to study each case (see Materials and Methods). When Mg(1) was bound at the G₂₇₄ phosphate group, no base pair breaking in the stem region was observed. However, the four bases in the SCS turned in and formed HB interactions. The RMSD of the hairpin loop was measured as the lowest ($3.4 \pm .2$ Å) in the subtype-A test group, while the overall RMSD was slightly larger than that of the subtype-B monomers (see Table S2).

When the Mg(1) ion was bound to the G₂₇₃ phosphate group, it showed how the magnesium ion binding site in subtype-A could significantly affect the stability of the structure. As shown in Figure 3(f), the monomer had a minor deformation from the initial structure. The overall RMSD in this case showed the lowest value ($3.5 \pm .3$ Å) in the subtype-A test group. In addition, it was as low as that of the subtype-B NMR and X-ray structures in the high salt concentration including magnesium ions. The presence of the Mg(1) at the G₂₇₃ phosphate group induced the P(267)–P(278) distance to have a short value (12.2 ± 1.6 Å) and the hairpin loop shape ratio became 2.2. All bases in the SCS bulged out and only C₂₇₉ formed HB interactions with backbone phosphate groups. Therefore, the results of our previous two MD simulations showed strong correlations with experimental results i.e. magnesium binding at the G₂₇₃ or G₂₇₄ phosphate groups is required for the dimerization of the subtype-A monomers (Jossinet et al., 1999).

The dependence of stability on salt concentrations

Figure 4 shows the RMSDs of the monomer, stem, and hairpin loop backbones in the subtype-A and -B test groups as a function of salt concentrations. As the salt concentration increased and when magnesium ions were present, the overall RMSD in both subtypes decreased. In the subtype-B NMR structure, the stem RMSD did not depend on salt concentrations, while the hairpin loop RMSD decreased at high salt concentrations. When magnesium ions were present, the subtype-B X-ray structure (1XPE) had the lowest stem RMSD due to the presence of a Mg(1)–(H₂O)₆ complex at the major groove. In the case of subtype-A, the stem was significantly distorted in 0 M and high salt concentrations, and had the two highest RMSD values between the test groups. At the low salt concentration, the hairpin loop showed the largest RMSD between the test groups. When magnesium ions were present, the large deformations in the stem region disappeared because the presence of Mg(H₂O)₆ complexes in the major groove contributed to the stability of the stem. When the Mg(1) ion bound to the G₂₇₃ phosphate site, the monomer structure had the lowest overall RMSD in the subtype-A test group. We also computed GDT_TS values between the initial and the average structures under different salt conditions (see Table S5). At 0 M salt concentration, due to the large deformation of the monomer structure, the GDT_TS values of the subtype-A and -B monomers were less than 10 and 30%, respectively. However, in the presence of magnesium, the GDT_TS value of the subtype-B monomer became 73%. When a magnesium ion was bound at the G₂₇₃ phosphate group, the GDT_TS value of the subtype-A also became 60%. Thus, both the RMSD and GDT_TS measurements indicate the

importance and relevance of ion concentration and magnesium binding in maintaining the proper monomer conformation.

One of the limitations of using RMSD and GDT_TS values is that they do not provide information about individual nucleotide behaviors. In order to determine how the deformation of each nucleotide can affect the conformation of the hairpin loop, we used the DPM (Parisien, Cruz, Westhof, & Major, 2009). With this method, it is possible to estimate the details of the deformation based on the similarity between an average structure of a MD trajectory and the corresponding initial structure. The DPM of each monomer under different salt concentrations was plotted in Figure 5. For example, the first row in the DPM plot was obtained by superimposing the first nucleotide (C₂₆₅) of the average structure on the first nucleotide (C₂₆₅) of the corresponding initial structure and measuring the distance of each corresponding nucleotide between the average and the initial structure. Therefore, low DPM values indicate high similarity between two structures while high DPM values imply low similarity due to a deformation. Each corner in DPM plot is the similarity measurement of stem regions between the initial structure and corresponding average structure. On the other hand, a vertical band in the middle of the DPM plot indicates similarity measurements of the hairpin loop with respect to superposition on the stem region. A horizontal band in the middle of DPM plot is the similarity measurements of the stem region with respect to superposition on the hairpin loop.

For 0 M salt concentration, the DPM plot of the sub-type-B NMR structure (Figure 5(a)) has large values in the vertical and horizontal bands in the middle of the plot, which indicates a large distortion of the SCS region. However, small variations in each corner indicate strong similarity of the stem region. As the salt concentration increased, the distortion in the SCS decreased and the effect of their superposition on the rest of the structure was reduced. A similar DPM was observed in the subtype-B X-ray structure (see supplementary material, Figure S2). Therefore, both the subtype-B NMR and the X-ray monomers had strong similarity between the initial and corresponding average structures at the high salt concentration including in the presence of magnesium. In subtype-A, wide distributions of high DPM values at the 0 M and high salt concentrations (see Figure 5(f) and (h)) were caused by the large deformations in the stem regions. When the Mg(1) ion was bound to the G₂₇₃ phosphate group, the overall DPM showed the strongest similarity in the subtype-A test group (Figure 5(k)). In addition, compared to the DPM values of the hairpin loop regions in Figure 5(j), where Mg(1) was bound to the G₂₇₄ phosphate group, the same hairpin loop region as seen in Figure 5(k) has lower DPM values due to the a smaller distortion. Thus, the subtype-A monomer had stronger similarity to the initial subtype-A structure in both the stem and the hairpin loop when Mg(1) was bound to G₂₇₃. This result supports the previous experimental results that G₂₇₃ is a crucial determinant of metal binding (Jossinet et al., 1999).

Comparison with mutated subtype-B NMR structure

When the experimental protocol in which the wild-type subtype-B dimerizes (Girard et al., 1999) is applied to the mutated subtype-B monomers (1JTJ), the mutated nucleotide (A₂₇₆) in the SCS prevents homodimerization and they remain as monomers (Kieken et al., 2002).

The mutated monomer may have an appropriate conformation for the dimerization, since when compared to the wild-type subtype-B monomer (2F4X), the stem helix of the mutated monomer is well maintained and five bases in the SCS are turned out for kissing loop formation. We compared the wild-type monomers which were obtained from our MD simulations at different salt concentrations with the mutated NMR monomer structure (1JTJ).

As shown in the Figure 6, the wild-type subtype-B monomer (NMR, 2F4X) at 0 M and low salt concentration had large RMSDs with respect to 1JTJ due to the deformation of the hairpin loop structure. However, the initial wild-type monomer and the monomer at high salt concentration showed relatively small RMSD values in the hairpin loop and overall regions when compared to 1JTJ. The overall structure of wild-type monomer at high salt concentration in Figure 2(d) also shows a similar shape when compared to the mutated monomer. In addition, most SCS bases in both the mutated and wild-type monomer at high salt concentration turned out and were available for kissing loop formation.

The wild-type subtype-B (X-ray) and -A monomers were also compared with the mutated monomer. In the subtype-B (X-ray) test groups, the RMSD of the wild-type subtype-B (X-ray) monomer at 0 M salt concentration was 7.1 Å with respect to the mutated one, while those of the monomers at high salt and in the presence of magnesium were 5.3 and 5.2 Å, respectively. In the subtype-A test group, the smallest RMSD value (5.7 Å) was obtained when a magnesium ion was bound to G₂₇₃, while when a magnesium ion was bound to G₂₇₄, its RMSD with respect to the mutated monomer was 6.2 Å. On the other hand, the RMSD at high salt concentration in the absence of magnesium was 9.7 Å due to distortions in the stem. The large distortion of the subtype-A monomers thus causes failure of dimerization at high salt concentration as indicated by experiment (Lodmell et al., 1998). Therefore, the dimerization of both subtypes may require the appropriate conformation of the monomer structures which is induced by the proper salt concentration and magnesium binding. In addition, as described below, the flanking bases in the subtype-A and -B appear to have different roles in determining the conformation of each monomer structure.

The role of flanking bases at the high salt concentration

In order to explain the different behaviors between the subtype-A and -B monomers in the high salt concentration, we monitored the role of the flanking bases in terms of HB interactions and the presence of ions near the hairpin loop. The flanking bases in the subtype-B monomers were found to support the appropriate hairpin loop shape by HB interactions with water molecules or a non-Watson–Crick HB interaction. However, in the subtype-A monomer, as G₂₇₃ formed multiple HB interactions with phosphate groups, subtype-A monomers experienced large deformations in the stem or the hairpin loop region regardless of the salt concentration. The detailed discussions are below.

In the subtype-B NMR structure, A₂₇₂ was completely turned in and formed a HB interaction with the phosphate group of C₂₇₅, while A₂₇₃ remained pointing out (see Tables S3 and S4). The other flanking base, A₂₈₀ also formed a weak HB with the C₂₇₅ phosphate group. These two flanking bases, A₂₇₂ and A₂₈₀ provided a structure that allowed Na⁺ ions to stay between them and the nearby backbone with high occupancy. For 50–100 ns time

ranges, one or two Na⁺ ions repeatedly populated the vicinity between the hairpin loop phosphate groups and the A₂₇₂ and A₂₈₀ bases for a time period of 2.0–5.0 ns. These Na⁺ ions interacted with two or three nearby water molecules and built stable HB interaction bridges. However, in the MD simulations of the other monomers (1XPE and 1XPF), the occupancies of HB interactions between the hairpin loops and waters mediated by Na⁺ ions occurred less than .5 ns. Therefore, these continuous HB interaction bridges may help to maintain the appropriate shape of the hairpin loops. In the subtype-B X-ray structure (1XPE), both flanking bases, A₂₇₂ and A₂₇₃ were turned in and A₂₇₂ formed a stable HB interaction with A₂₈₀ (see Table S4). This non-Watson–Crick HB interaction may also help to maintain the appropriate shape for the hairpin loop.

In the X-ray structure of subtype-A, both flanking bases pointed in within 4 ns in the high salt concentration. During the period of 23–38 ns, G₂₇₃ formed multiple HB interactions including H21, H22 (273)...O1P, O2P (276), H1 (273)...O1P (277), and H21 (273)...O2P (277) (see Figure 7(a)). These multiple HB interactions sharply bent the hairpin loop curvature along G₂₇₃–G₂₇₄–U₂₇₅–G₂₇₆–C₂₇₇ and constrained its shape. At 38 ns, the HB interactions between the G₂₇₃ and C₂₇₇ phosphate groups were broken and simultaneously the stem helix started to unwind. Once the stem helix was completely unwound, G₂₇₃ recovered the HB interactions with the C₂₇₇ phosphate group again. Therefore, the strong multiple HB interactions of G₂₇₃ with nearby phosphate groups may cause a large deformation of the overall RNA helix.

Furthermore, G₂₇₃ was also found to contribute to the deformation of subtype-A monomers at the other two salt concentrations. Under low salt concentration, the back-bone along A₂₇₂–C₂₇₇ rapidly deformed due to the strong electrostatic repulsion at the beginning of the MD simulation. As the hairpin loop elongated, G₂₇₃ had more chance to form HB with the lower hairpin loop structure (277–279). G₂₇₃ formed H21 (273) O2P (279), H22 (273) O2P (280), and H1 (273) O2P (279) at 40 ns and these HB interactions locked the G₂₇₃ base and the C₂₇₉ back-bone for the rest of the MD simulation (see Figure 7(b)). In addition, these HB interactions caused the largest RMSD of the hairpin loop in the entire test group by inducing U₂₇₅–A₂₈₀–C₂₇₉ to stack and point in and G₂₇₆–C₂₇₇–A₂₇₈ to stack and point out. In the 0 M salt concentration, G₂₇₃ formed H21 (273)...O2P (280) and H1 (273) O2P (280) at 9 ns and as these HBs broke, the stem helix started to unwind.

The role of magnesium ions in the subtype-A monomer

When Mg(1) and Mg(2) bound to the phosphate groups of G₂₇₄ and G₂₇₆, respectively, the water molecules of Mg(2)–(H₂O)₅ at the G₂₇₆ phosphate group formed HB interactions with U₂₇₀ and G₂₇₁. These HB interactions interfered with the G₂₇₁–C₂₈₁ base pair and eliminated these base pair after 35 ns. Then, G₂₇₁ formed a stable HB with A₂₇₂ (H22 (271)...N1 (272)) for 40–100 ns (see Table S4), while HB interactions between G₂₇₁ and the water molecules of Mg(2)–(H₂O)₅ remained until 60 ns. Therefore, the bound magnesium ion at the G₂₇₆ phosphate group induced the breaking of the base pairs in the stem region and distorted the hairpin loop structure.

When Mg(1) bound to the G₂₇₄ site, the Mg(1)–(H₂O)₅ complex prevented G₂₇₃ from forming HB with the upper hairpin loop backbone (274–276) at the beginning of the MD

simulation. At 35 ns, the water molecules in the $\text{Mg}(1)\text{--}(\text{H}_2\text{O})_5$ complex formed HB interactions with the U_{275} phosphate group and prevented the hairpin loop structure from further deformation (see Figure 7(c)). Therefore, the hairpin loop RMSD had the lowest value in the subtype-A test group. When $\text{Mg}(1)$ bound to the G_{273} phosphate group, the entire monomer showed the most stable dynamics in the subtype-A test group. Water molecules in the $\text{Mg}(1)\text{--}(\text{H}_2\text{O})_5$ complex formed very stable HB interactions with U_{275} and the G_{276} phosphate group and induced G_{274} , U_{275} , and G_{276} to point out. Once the upper hairpin loop backbone was locked by the $\text{Mg}(1)\text{--}(\text{H}_2\text{O})_5$ complex, it provided a stable scaffold so that the $\text{Mg}(\text{A}_1)\text{--}(\text{H}_2\text{O})_6$ complex, which contained one of the magnesium ions added to the system to increase the MgCl_2 concentration, stayed between the stem and the lower hairpin loop backbone (see Figure 7(d)). Water molecules from the $\text{Mg}(\text{A}_1)\text{--}(\text{H}_2\text{O})_6$ complex formed HB interactions with the nearby backbone and bases for 24–100 ns. These HB networks stabilized the lower hairpin loop structure and induced C_{277} and A_{278} to point out. Furthermore, the $\text{Mg}(1)\text{--}(\text{H}_2\text{O})_5$ complex prevented the G_{273} base from forming HB interactions for the entire 100 ns MD simulation by causing it to point out.

Examination of the role of salt strength and magnesium binding by applying restraints

It was found that Coulombic repulsion between nucleotides caused tethered duplexes to extend under low salt concentrations, while high salt concentrations induced relaxation of the electrostatic repulsion (Bai, Das, Millett, Herschlag, & Doniach, 2005). In a similar manner, our MD simulation results showed that high salt concentration played the role of maintaining the appropriate hairpin loop shape by reducing the electrostatic repulsion between the stem and the hairpin loop. In order to verify this, we applied a parabolic restraint on the distance between P (267) and P(278) ($20.0 \text{ \AA}$, $10 \text{ kcal}/(\text{mol}\text{\AA})$), see Figure 1(e)) in the subtype-B NMR structure under 0 M salt concentration. Figure 2(e) shows the average structure of the restrained monomer. The hairpin loop maintained its curvature for the entire simulation and most bases in the SCS pointed out and stacked together. The overall structure showed minor distortion and the average RMSD and the DPM plot were as low as that of 2F4X and 1XPE in the high salt concentrations (see Table S2 and Figure 5(d)).

The effect of high salt concentration was also tested by removing the applied distance restraint. For this test, we used the structure at 60 ns where the distance restraint was applied, and we performed an additional 60 ns MD simulation without applying the distance restraint. As shown in Figure 2(f), the average structure of the monomer deformed to a structure that was similar to what was observed in the 0 M salt and in the low salt concentrations. The P(267)–P(278) distance increased to $28 \text{ \AA}$ and the overall RMSD reached $7.0 \pm .7 \text{ \AA}$. As the hairpin loop deformed, most bases in the SCS pointed in while flanking bases pointed out. The DPM (Figure 5(e)) also showed that the overall structure returned to that of the 0 M salt concentration when the restraint was released.

In the subtype-A monomer, G_{273} was considered to perform a major role in distorting the stem and the hairpin loop in the absence of magnesium ions. In addition, the most stable subtype-A monomer was obtained when a magnesium ion was bound to the G_{273} phosphate site which prohibited the G_{273} base from forming any HB interaction. In order to test the role of G_{273} , we applied three parabolic restraints to the subtype-A monomer at the 0 M salt

concentration. The effect of high salt concentration was mimicked by applying a distance restraint between P(267) and P(278) (20.0 Å, 50 kcal/(molÅ), see Figure 1(e)). Two additional restraints, a torsion restraint (N1(271)–P(271)–P(273)–N1(273), $225^\circ < \Phi_{\text{Torsion}} < 255^\circ$, 10 kcal/(molÅ)) and an angular restraint (P(271)–C4'(273)–N1(273), $85^\circ < \Theta_{\text{Angle}} < 105^\circ$, 50 kcal/(molÅ)) (see Figure 1(E)) were applied based on the corresponding angles in the initial X-ray structure, 240.7° for N1(271)–P(271)–P(273)–N1(273) and 97.8° for P(271)–C4'(273)–N1(273). As a consequence of these restraints, G₂₇₃ remained pointing out and it was prohibited from forming multiple HB interactions with the G₂₇₆ and the C₂₇₇ phosphate groups. For the 100 ns MD simulation, the P(267)–P(278) distance was maintained at 18.2 ± 1.9 Å and the two angles, P(271)–C4'(273)–N1(273) and N1(271)–P(271)–P(273)–N1(273) were maintained at $91.1 \pm 6.2^\circ$ and $236.0 \pm 16.9^\circ$, respectively. Compared to the average structures at 0 M salt concentration without restraints (Figure 3(a)), the average structure's shape with the three restraints applied was well maintained (Figure 3(g)). The overall RMSD was two times smaller than those of the 0 M and high salt concentrations, and the hairpin loop RMSD was 1.7 times smaller than that found with low salt concentration. Although G₂₇₃ maintained its original orientation, G₂₇₄ flipped over and formed weak HB interactions with A₂₇₂ and G₂₇₃. Compared to the DPM in 0 M salt concentration (Figure 5(f)), the dissimilarity in the stem and hairpin loop regions from the X-ray structure significantly disappeared (Figure 5(l)).

The role of G₂₇₃ was verified again by releasing all restraints from the structure at 50 ns in the previous simulation. After the restraints were released, A₂₇₂ and G₂₇₃ became more flexible and the two angles, P(271)–C4'(273)–N1(273) and N1(271)–P(271)–P(273)–N1(273) changed to $70.2 \pm 9.5^\circ$ and $291.6 \pm 11.1^\circ$, respectively. The P(267)–P(278) also increased to 31.0 ± 2.2 Å. Due to these flexibilities, A₂₇₂ and G₂₇₃ formed stronger HB interactions with G₂₇₄ than the previous simulation and induced distortions in the hairpin loop structure. Strong HB interactions in A₂₇₂ and G₂₇₃ also caused most of bases in the hairpin loop structure to point in and start to form HB interactions. Due to these events, the loop shape ratio decreased to .7 and the overall RMSD increased to $7.7 \pm .6$ Å. The average structure after removing the restraints showed a large deformation from the initial X-ray structure (Figure 3(h)) and its DPM plot showed that the overall structure returned to that of the 0 M salt concentration (Figure 5(f) and (m)).

Conclusions

We investigated the conformational change of the HIV-1 subtype-A and -B monomers as a function of salt concentration and magnesium binding. For both sub-types, our MD simulation results showed strong correlations with various experimental results. It was experimentally shown that dimerization of the subtype-B monomer occurs at high salt concentration or in the presence of magnesium, in which case it does not require magnesium binding, while the subtype-A monomers require magnesium binding at the G₂₇₃ or G₂₇₄ phosphate groups for dimerization (Jossinet et al., 1999; Lodmell et al., 1998). At low salt concentration, we found that monomer structures of both subtypes were significantly distorted during MD simulations. In high salt concentration and in the presence of magnesium ions, our MD simulation showed that the sub-type-B monomers maintained their conformations without magnesium binding, while the subtype-A monomer was significantly

distorted in the high salt concentration. The subtype-A monomer maintained the appropriate conformations only when a magnesium ion was bound to the G₂₇₃ or G₂₇₄ phosphate groups. Therefore, the subtype-B monomer has a conformation that is compatible with dimerization under high salt or high salt in the presence of magnesium, while the sub-type-A monomer has a conformation that is compatible with dimerization when magnesium is bound at the G₂₇₃ or G₂₇₄ phosphate groups. In addition, we found that the flanking bases play important roles for inducing these conformations.

Based on an N1-methylation experiment, it was found that non-Watson–Crick interactions between A₂₇₂ and A₂₈₀ were not important for dimerization (Jossinet et al., 1999). In our MD simulations, the HB interaction analysis of the flanking bases showed that the flanking bases in the subtype-B can stabilize the hairpin loop structure with either non-Watson–Crick interactions or water bridges between them. In the case of subtype-A, the flanking base G₂₇₃ contributed to a significant deformation in the monomer structure by forming multiple HB interactions with the upper or lower hairpin loop phosphate groups. Due to the deformation of the hairpin loop, a non-Watson–Crick interaction between A₂₇₂ and A₂₈₀ was not observed in low and high salt concentrations.

Although previous Brownian simulations indicate that the G₂₇₃ or G₂₇₄ phosphate groups in the subtype-A have a high probability for magnesium binding (Jossinet et al., 1999), it was unclear as to what the role of the bound magnesiums were at these phosphate groups. Based on our MD simulations, we suggest that a magnesium ion bound at the G₂₇₃ or G₂₇₄ phosphate groups can significantly affect the conformation of the subtype-A monomer. Especially, when the Mg(1) ion was bound to the G₂₇₃ phosphate group, the subtype-A monomer experienced the minimum deformation and five bases in the SCS pointed out for potential kissing loop formation. The previous experiment also showed that G₂₇₃ was a crucial determinant of metal binding (Jossinet et al., 1999). In addition, even though the subtype-A dimer has weaker kissing loop interactions due to the two A–U and four C–G base pairs rather than the six C–G base pairs found in subtype-B (see Figure 1(a)), our results showed that the stability of the subtype-A monomer was determined by the behavior of the flanking G₂₇₃ base and magnesium binding at the G₂₇₃ phosphate group. The X-ray structure (1XPF) also indicates that there is no magnesium ion in the vicinity of A₂₇₅ or U₂₇₈. Therefore, the requirement of magnesium binding in the subtype-A dimerization, which stabilizes the subtype-A monomer structure, seems to be independent of SCS sequence.

In conclusion, based on the strong correlations between our MD simulations and experimental results (Jossinet et al., 1999; Kieken et al., 2002; Lodmell et al., 1998), we suggest that the kissing loop formation of HIV-1 DIS may require the proper conformation of the monomers which is induced by the appropriate salt strength and magnesium ion binding. In addition, since our results show that the conformation of the hairpin loop can be affected by the flanking bases, salt concentration, and magnesium binding, these relationships can be used for developing antiretroviral therapy and designing self-assembling RNA nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HIV	human immunodeficiency virus
MD	molecular dynamics
DIS	dimerization initiation site
GDT_TS	Global Distance Test Total Score
SCS	self-complementary sequence
HB	hydrogen bond
DPM	deformation profile matrix
SELEX	systematic evolution of ligands by exponential enrichment
PDB	Protein Data Bank

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Figure 1.

a) Secondary structure of subtype-A and subtype-B. “*” indicate the counterpart monomer of the kissing loop complex. Three nucleotides in the subtype-B monomer (red colored) are different from the subtype-A monomer. (b) the X-ray structure of subtype-A (1XPF), (c) subtype-B (1XPE) and (d) NMR structure of subtype-B (2F4X). (e) Distance and torsion restraints are indicated by the solid red arrows. Angle restraint is indicated by the solid blue arrow. The horizontal loop component, (N1(271)–P(277)) and the vertical loop component, (P(273)–P(280)) are indicated by the dashed black arrows.

Figure 2.

Green colored structure is the original NMR structure of subtype-B (2F4X). Orange colored structures are average monomer structures of subtype-B at (a) 0 M, (b) low salt and (c) high salt concentrations. (d) is comparison between 1JTJ (model 2, blue colored structure) and wild-type average structure in high salt concentration. (e) is the average structure of subtype-B when a distance restraint is applied and (f) is the average structure when the distance restraint is released. Average structure at 0 M salt concentration is obtained from 20 to 50 ns and the other average structures are obtained from 50 to 100 ns. All structures are aligned along residue C₂₆₅ to residue G₂₇₁.

Figure 3.

Green colored structure is the original X-ray structure of subtype-A (1XPF). Orange colored structures are average structures of (a) 0 M concentration, (b) low salt concentration, (c) high salt concentration, (d) high salt concentration with magnesium ions bound to the G_{274} and G_{276} phosphate groups, (e) high salt concentrations with a magnesium ion bound to the G_{274} phosphate group, (f) high salt concentrations with a magnesium ion bound to the G_{273} phosphate group, (g) 0 M salt concentration with three restraints and (h) 0 M salt concentration after releasing three restraints. Orange spheres are the average locations of bound magnesium, Mg(1) and Mg(2). Average structure at 0 M salt concentration is obtained from 20 to 50 ns and the other average structures are obtained from 50 to 100 ns. All structures are aligned along residue C_{265} to residue G_{271} .

Figure 4.

RMSD variation of (a) subtype-B NMR, (b) subtype-B X-ray and (c) subtype-A X-ray structures as a function of salt concentration and magnesium ion binding. (*): Mg(1) and Mg(2) bound at the G₂₇₄ and G₂₇₆ phosphate groups. (**): Mg(1) bound at the G₂₇₄ phosphate group. (***): Mg(1) bound at the G₂₇₃ phosphate group.

Figure 5.

DPM plots of the subtype-A and -B monomers. The subtype-B NMR monomers with different salt concentrations and restraint are plotted in (a)–(e). The subtype-A monomers with different salt concentrations and restraints are plotted in (f)–(m).

Figure 6. RMSD of the wild-type subtype-B monomers (2F4X) at different salt concentrations with respect to 19 NMR structures in the mutated subtype-B monomer (1JTJ). (a) is the overall, (b) is the stem region, and (c) is the hairpin loop RMSD.

Figure 7.

The HB interactions of G₂₇₃ in (a) high salt concentration, (b) low salt concentration. (c) When Mg(1) binds to G₂₇₄, a water molecule in the Mg(1)–(H₂O)₅ complex formed stable HB interactions with the U₂₇₅ phosphate group. (d) When Mg(1) binds to G₂₇₃, water molecules in Mg(1)–(H₂O)₅ complex formed stable HB interactions with U₂₇₅ and G₂₇₆ phosphate groups. Water molecules in the Mg(A₁)–(H₂O)₆ complex also formed stable HB interaction with nearby bases and C₂₇₇ and A₂₇₈ phosphate groups. Green spheres indicate magnesium ions. For simplicity, some bases are removed from figures.