A Thermodynamic Investigation into the Stabilization of Poly(dA)·[poly(dT)]₂
Triple Helical DNA by Various Divalent Metal Ions

Byung-Hoon Choi, Ga Young Yeo, Jinah Jung, Bae Wook Lee, Sung Wook Han,† and Tae Sub Cho*

Department of Chemistry, Yeungnam University, Gyeongsan City, Gyeongbuk 712-749, Korea. †E-mail: tscho@yu.ac.kr

School of Herb Medicine Resources, Kyungwoon University, Gumi, Gyeongbuk 730-739, Korea

Received June 16, 2009, Accepted September 29, 2009

Effects of representative group II and transition metal ions on the stability of the poly(dA)·[poly(dT)]₂ triplex were investigated by the van’t Hoff plot constructed from a thermal melting curve. The transition, poly(dA)·poly(dT)₂ → poly(dA)·poly(dT) + poly(dT), was non-spontaneous with a positive Gibbs’s free energy, endothermic (ΔH° > 0), and had a favorable entropy change (ΔS° > 0), as seen from the negative slope and positive y-intercept in the van’t Hoff plot. Therefore, the transition is driven by entropy change. The Mg²⁺ ion was the most effective at stabilization of the triplex, with the effect decreasing in the order of Mg²⁺ > Ca²⁺ > Sr²⁺ > Ba²⁺. A similar stabilization effect was found for the duplex to single strand transition: poly(dA)·poly(dT) → poly(dA) + 2poly(dT), with a larger positive free energy. The transition metal ions, namely Ni²⁺, Cu²⁺, and Zn²⁺, did not exhibit any effect on triplex stabilization, while showing little effect on duplex stabilization. The different effects on triplex stabilization between group II metal ions and the transition metal ions may be attributed to their difference in binding to DNA; transition metals are known to coordinate with DNA components, including phosphate groups, while group II metal ions conceivably bind DNA via electrostatic interactions. The Cd²⁺ ion was an exception, effectively stabilizing the triplex and melting temperature of the third strand dissociation was higher than that observed in the presence of Mg²⁺, even though it is in the same group with Zn²⁺. The detailed behavior of the Cd²⁺ ion is currently under investigation.

Key Words: Triplex DNA, Divalent ions, Thermodynamics, Enthalpy, Entropy

Introduction

Since the first triplex helical nucleic acids were reported in 1957,¹ in which a 2 : 1 ratio of poly(U) and poly(A) was shown to form a stable complex, they have been widely studied for their high selectivity of the third strand, for recognizing a single site in large duplex DNA, and for providing potentials for biological and therapeutic applications that include inhibition of gene expression and design of artificial nucleases.² The discovery of an intramolecular triplex (H-DNA) within a supercoiled plasmid highlighted the biological importance of the triple helical DNA.³,⁴ In the case of DNA, poly(dA)·poly(dT) and poly(dG)·poly(dC) can form a triple helix with poly(dT) and poly(dC), respectively, in the presence of divalent Mg²⁺ ions.¹ In the triple helix, the third poly(dT) and poly(dC) strand locate in the major groove of the template duplex and form Hoogsteen type hydrogen bonds with the template polypurimidine strand. In general, the third Hoogsteen base-paired strand is less strongly bound to the duplex than the two Watson-Crick bound strands of the corresponding duplex.

The presence of divalent ions, particularly Mg²⁺, is crucial in the structure and biological activity of biomolecules, including nucleic acids and various proteins. Especially in the stabilization of the triplex nucleic acids, the presence of the Mg²⁺ ion is essential. The role of the Mg²⁺ ion in the stabilization of the triplex RNA has been investigated.⁵,⁶ It was proposed that RNA stabilization arises from two energetically distinct modes of Mg²⁺ binding, namely diffuse- and site-binding.⁷ The Mg²⁺ ions are attracted electrostatically to the strong anionic field surrounding the RNA in a diffusive binding mode, while they bind at the specifically arranged electronegative ligands. However, the role of the Mg²⁺ ions in the stabilization of the nucleic acids has not been fully elucidated. The divalent metal ions belonging to the d-block, such as Co²⁺, Ni²⁺, and Zn²⁺, exhibited contrasting interactions with DNA.⁸ For instance, the Zn²⁺ ion is believed to coordinate with negatively charged phosphate groups at low or intermediate concentrations while inducing aggregation of DNA or formation of M-DNA at a high concentration.⁹

In order to understand the role of divalent ions, including Mg²⁺, in the stabilization of triplex DNA, the thermodynamic aspects of the concentration-dependent thermal melting profile of poly(dA)·[poly(dT)]₂ triplex DNA, in the presence of various alkaline-earth metals (Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺) and transition metal ions (Ni²⁺, Cu²⁺, Zn²⁺, Cd²⁺) were investigated.

Experiment

Formation and dissociation of the poly(dA)·[poly(dT)]₂ triplex. Poly(dA) and poly(dT) were purchased from Sigma and dissolved in a 5.0 mM cacodylate buffer solution, pH 7.0, containing 10 mM NaCl. The triplex poly(dA)·[poly(dT)]₂ was obtained by heating a 2 : 1 molar ratio of poly(dT) and poly(dA) in the presence of the desired concentration of the metal ion, followed by overnight annealing at room temperature. The concentrations of polynucleotide were measured spectrophotometrically using the extinction coefficients of ε₂⁵⁷nm = 8600 M⁻¹cm⁻¹ and ε₂⁶⁴nm = 8520 M⁻¹cm⁻¹ for poly(dA) and poly(dT), respectively. The thermal melting profile was
and single-strand are the same, being \( (1 - \alpha)^2 \), and that of the third strand is \( \frac{aC_t}{2} \), where \( C_t \) is the total concentration of poly(dA) · poly(dT) and poly(dT) available to form triplex poly(dA) · poly(dT) · poly(dT). Thus, the observed equilibrium constant is given by:

\[
K_{eq} = \frac{[D][S]}{[T]} = \frac{(1 - \alpha)^2 C_t}{2\alpha}
\]

The temperature-dependent equilibrium constant is related to the thermodynamic parameters, \( \Delta H^o \) and \( \Delta S^o \), through the well-known van’ Hoff equation:

\[
\ln K_{eq} = -\frac{\Delta H^o}{R} \left( \frac{1}{T} \right) + \frac{\Delta S^o}{R}
\]

where \( R \) is the gas constant (1.987 cal·mol\(^{-1}\)·K\(^{-1}\)) and \( T \) is the absolute temperature. The Gibb’s free energy for the transition can easily be calculated from the equation:

\[
\Delta G^o = -RT \ln K_{eq} = \Delta H^o - T \Delta S^o
\]

The second transition that occurs at higher temperature corresponds to the transition from the double stranded poly(dA)-poly(dT) and poly(dT) to the three single-stranded polynucleotides:

\[
\text{poly(dA)} \text{poly(dT)} + \text{poly(dT)} \rightleftharpoons \text{poly(dA)} + 2 \text{poly(dT)}
\]

In this case, the equilibrium constant is given by:

\[
K_{eq} = \frac{2(1 - \alpha)^2 C_t}{3\alpha}
\]

Results and Discussion

Melting profile and thermodynamic parameters in the presence of Mg\(^{2+}\) ions. The temperature-dependent absorbance change of the poly(dA) · poly(dT) · poly(dT) triplex at 260 nm in the presence of various concentrations of Mg\(^{2+}\) ion is depicted in Fig. 1(a). As the temperature increases, the dissociation of the triplex poly(dA) · poly(dT) · poly(dT) exhibited two transitions, except for the lowest Mg\(^{2+}\) ion concentration (40 µM). Therefore, it is clear that the Mg\(^{2+}\) ion concentration required to stabilize the 30 µM nucleobase concentration (or 10 µM base triplet) of the poly(dA) · poly(dT) · poly(dT) triplex is above 40 µM. When the Mg\(^{2+}\) ion concentration reached 80 µM, the melting profile started to exhibit two transitions. The melting temperatures of both transitions that corresponded to the transition from triplex to duplex, plus one single strand (\( T_{m1} \)), and that from duplex to two single-stranded DNAs (\( T_{m2} \)), increased with the Mg\(^{2+}\) ion concentration.

The melting temperatures of both transitions can be seen more clearly when the change in absorbance per change in the temperature, a differential of absorbance relative to the temperature, is plotted with respect to temperature (Fig. 1b). As can be seen clearly, \( T_{m1} \) increases as the Mg\(^{2+}\) ion concentration increases, being 37.9 °C at 80 µM Mg\(^{2+}\) and 53.2 °C at 200 µM, while the values of \( T_{m2} \) were observed at 65.3 and 69.8 °C at the same Mg\(^{2+}\) concentrations (Table 1). From the slope and y-intercept of the van’t Hoff plot (Fig. 2),
the entropy and enthalpy change for the dissociation of the triplex and duplex can be calculated. The values are summarized in Table 1. However, it has to be noted that the thermodynamic values calculated here from the observed absorbance by the conventional spectrophotometer may not be sufficiently accurate (may contain some degree of error) because the temperature range adopted in this work is very narrow (typically 2 ~ 3 degrees), and the change in enthalpy was assumed to be constant at the given temperature range. The latter limitation can be seen particularly in the dissociation of the duplex, as the van’t Hoff plot deviated somewhat from a straight line. Nevertheless, thermodynamic parameters obtained from these measurements may provide some clues for the metal ion-DNA interaction. As seen in Fig. 2, the slope of the van’t Hoff plot for both transitions is negative and the y-intercept are positive, suggesting that both transitions are endothermic and entropically favorable. The thermodynamic parameters obtained from the slope and y-intercept are summarized in Table 1. In the case of the triplex to duplex plus single-stranded transition, the unfavorable enthalpy showed the tendency to increase, being 82.5 Kcal·mol⁻¹ in the presence of 80 µM Mg²⁺ and 148.7 Kcal·mol⁻¹ at 200 µM, suggesting that in the presence of higher concentrations of the Mg²⁺ ions, the Hoogsteen paired third poly(dT) strand is more stable. In the range of the concentration investigated in this work, no saturation of Mg²⁺ ions was found, implying that not all the added Mg²⁺ participates the third strand stabilization. The entropy change increases with increasing Mg²⁺ ion concentration. Considering that increases in entropy change reflect the degree of disorder of the system, the triplex at a low Mg²⁺ ion concentration may be more flexible because the degree of disorder of the product, duplex plus single stranded poly(dT), is conceivably similar.

In the transition from duplex poly(dA)·poly(dT) plus poly(dT) to three single-stranded DNAs, as in the one poly(dA) and 2 poly(dT)s case, the values of the enthalpy and entropy and their dependency on Mg²⁺ concentration is clearly different from those observed for the triplex-duplex transition. The enthalpy change for the duplex to single-stranded DNA is similar or larger compared to that of the triplex to duplex transition at low Mg²⁺ concentrations (110.1 Kcal·mol⁻¹ in 40 µM Mg²⁺) while it is significantly lower at the Mg²⁺ concentration of 120 µM or above (Table 1, 94.2 Kcal·mol⁻¹ in 200 µM Mg²⁺). Although it is unclear, more Mg²⁺ ions are associated with the phosphate groups of the template duplex at a high Mg²⁺ ion concentration, which may affect the stability of the duplex. Entropy changes exhibited similar behavior, being favorable at low Mg²⁺ concentrations, when 40 or 80 µM of Mg²⁺ ions were present, and were higher compared to that in the presence of Mg²⁺ ion concentration higher than 120 µM. The Gibb’s free energy for the transition from poly(dA)·poly(dT) to poly(dA) and poly(dT) are independent of the Mg²⁺ concentration, ~13 Kcal·mol⁻¹ at 25 °C. Therefore, the overall transition is endothermic. In contrast, the transition from triplex to duplex, dissociation of the third strand, exhibited 5.1 Kcal·mol⁻¹ at 25 °C in the presence of 80 µM of Mg²⁺ ion. This value increased upon increasing the Mg²⁺ ion concentration. Therefore, it seems to be clear that the Mg²⁺ ions contributing to the stabilization of the triples is not saturated, even at 200 µM. It is further conclusive that the binding site of the Mg²⁺ ion that stabilizes the triplex is different from that for the duplex.

**Table 1.** Thermodynamic parameters for the transition: poly(dA)-[poly(dT)]; –→ poly(dA)-poly(dT) + poly(dT); –→ poly(dA) + 2poly(dT), in the presence of various concentrations of Mg²⁺ ions. ∆G was calculated from average enthalpy and entropy change.

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<td>404.6 ± 10.9</td>
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<tr>
<td>200</td>
<td>449.9 ± 3.6</td>
<td>14.6</td>
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</table>

**Figure 2.** Chosen examples of the van’t Hoff plot for the triplex to duplex transition (open symbols) and duplex to single strand transition (closed symbols) in the presence of 40 µM (circles), 120 µM (triangles), and 200 µM (squares) Mg²⁺.
Figure 3. The dA/dT plot of the dissociation of the poly(dA)·[poly(dT)]2 triplex in the presence of various divalent ions from group II elements. Panel a: 80 µM and panel b: 160 µM.

Figure 4. The dA/dT plot of the dissociation of the poly(dA)·[poly(dT)]2 triplex in the presence of various divalent transition metal ions. Those obtained in the presence of the Mg2+ ion (solid curve) are inserted for comparison. Panel a: 80 µM and panel b: 160 µM.

The Tm1 was not observed, even at 80 µM, suggesting that the ions in the higher periods are far less effective. The Tm2 corresponding to the dissociation of the duplex poly(dA)·poly(dT) was similar for Mg2+, Ca2+, and Sr2+ ions, being 65.3, 64.8, and 64.3 °C, respectively. The hypochromicities are also similar, suggesting that the effects in the stabilization of duplex poly(dA)·poly(dT) by these ions are similar, which is in contrast with the third strand case. The Tm2 in the presence of Ba2+ ions is somewhat lower, being 62.4 °C, and the hypochromicity is also lower.

At a concentration of 160 µM (Fig. 3b), all ions from group 2 exhibited two Tm values, ensuring the occurrence of two transitions, triplex to duplex and duplex to single strand DNA. However, the effects were different. As was discussed in the 80 µM case, the effect in stabilization of the duplex (Tm2) is similar for Mg2+, Ca2+, and Sr2+ ions, being 68.8, 68.3, and 68.3 °C, respectively, and slightly higher than those at 80 µM. The hypochromicity also appeared to be similar. The Ba2+ ion was the least effective (Tm2 = 66.6 °C), with the lowest hypochromicity, as observed at 80 µM. The observed Tm1 increased with increasing period, the order being: Mg2+ (50.3 °C) > Ca2+ (49.5 °C) > Sr2+ (42.7 °C) > Ba2+ (39.9 °C). The tendency of decreasing the stabilization effect with increasing period suggests that the size of the ion may play an important role in triplex stabilization. The Mg2+ ion may fit in a certain site to interact with the third poly(dT) strand in the triplex. Conversely, the Ba2+ ion is possibly too large to fit in the same site. Thermodynamic parameters in the triplex and duplex equilibrium, as well as that of the duplex and single strand, are in similar ranges in the accuracy of the method adopted in this work, whose limitation was discussed in the previous section. For all ions, the ΔH° values were positive for both the Tm1 and Tm2 transitions (endothermic transition), while ΔS° values were also positive (favorable). The measured values of the thermodynamic parameters (data not shown) fall into similar ranges with similar behavior in the Mg2+ ion case. For instance, the enthalpy change corresponding to the triplex to duplex transition at various ion concentrations was approximately 80 ~ 100 Kcal·mol⁻¹ for Ca2+ ion, 70 ~ 130 Kcal·mol⁻¹ for Sr2+, and 60 ~ 90 Kcal·mol⁻¹ for Ba2+ ion, with Ba2+ being the lowest. This corresponds to the dissociation of the duplex at 80 ~ 100, 90 ~ 140, and 90 ~ 120 Kcal·mol⁻¹, respectively, for Ca2+, Sr2+, and Ba2+ ions. The entropy changes also fall into the similar range. The Gibb’s free energy at 25 °C for the triplex to duplex transition was 4.5, 5.3, 9.5, and 9.6 Kcal·mol⁻¹, respectively, in the presence of 80, 120, 160, and 200 µM of Ca2+ ion, while it was 13.7 ± 2.0 Kcal·mol⁻¹, being independent of the ion concentration for all Ca2+ concentrations. Other divalent ions from alkali-earth elements, namely Sr2+ and Ba2+, exhibited a similar tendency (data not shown), except for at an ion concentration of 80 µM, at which no triplex stabilization was observed.

Effect of transition metal ions in triplex stabilization. The thermal transition curve in the presence of various divalent transition metal ions, including Ni2+, Zn2+, and Cu2+, are com-
effects in stabilization of both the poly(dA)·[poly(dT)]2 triplex and the poly(dA)·poly(dT) duplex. As shown in Fig. 5 and Table 2, Tm1 was higher than that induced by the Mg2+ ion, being 39.0 and 52.4 °C at concentrations of 80 and 160 µM, respectively. However, Tm2 is lower at all concentrations. The hypochromism for triplex dissociation is lower for Cd2+ than for Mg2+ at all concentrations, while that for duplex dissociation by Cd2+ is lower at low ion concentrations and the same at high ion concentrations compared to that observed for the Mg2+ ion. The thermodynamic parameters for the triplex to duplex and duplex to single strand transitions at various Cd2+ concentrations are summarized in Table 2. As observed for Mg2+, the enthalpy changes appear to be unfavorable (endothermic), while entropy changes were favorable for both transitions. The values were in a similar range with the Mg2+ ion.

Although the Cd2+ ion belongs also to the transition metal group II, namely Mg2+, Ca2+, Sr2+, and Ba2+, coordinate with the DNA components, particularly with the negatively charged phosphate groups.7

The presence of divalent metal ions originating from group II, namely Mg2+, Ca2+, Sr2+, and Ba2+, stabilize the poly(dA)·[poly(dT)]2 triplex and poly(dA)·poly(dT) duplex. The positive Gibb’s free energy for both dissociations from triplex to duplex and duplex to single strand were established, indicating that the dissociations are non-spontaneous and driven by favorable entropy changes. Nevertheless, the divergent trans-
ion metal ions, namely Ni\textsuperscript{2+}, Zn\textsuperscript{2+}, and Cu\textsuperscript{2+}, were unable to stabilize the triplex and were far less effective in duplex stabilization compared to the Mg\textsuperscript{2+} ion. The Cd\textsuperscript{2+} ion, also a transition metal ion with the same electron configuration as Zn\textsuperscript{2+}, was an exception and was more effective in triplex stabilization and less effective in duplex stabilization compared to the Mg\textsuperscript{2+} ion.

References