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Hydration Regulates Thermodynamics of G-Quadruplex Formation under Molecular Crowding Conditions

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Abstract: The effect of molecular crowding on the structure and stability of biomolecules has become a subject of increasing interest because it can clarify how biomolecules behave under cell-mimicking conditions. Here, we quantitatively analyzed the effects of molecular crowding on the thermodynamics of antiparallel G-quadruplex formation via Hoogsteen base pairs and of antiparallel hairpin-looped duplex (HP duplex) formation via Watson−Crick base pairs. The free energy change at 25 °C for G-quadruplex formation decreased from −3.5 to −5.5 kcal mol^{-1} when the concentration of poly(ethylene glycol) 200 was increased from 0 to 40 wt %, whereas that of duplex formation increased from −9.8 to −6.9 kcal mol^{-1}. These results showed that the antiparallel G-quadruplex is stabilized under molecular crowding conditions, but that the HP duplex is destabilized. Moreover, plots of stability (ln K_{obs}) of the DNA structures versus water activity (ln a_w) demonstrated that the ln K_{obs} for G-quadruplex formation decreased linearly as the ln a_w increased, whereas that for duplex formation increased linearly with the increase in ln a_w, suggesting that the slope approximately equals the number of water molecules released or taken up during the formation of these structures. Thus, molecular crowding affects the thermodynamics of DNA structure formation by altering the hydration of the DNA. The stabilization of the DNA structures with Hoogsteen base pairs and destabilization of DNA structures with Watson−Crick base pairs under molecular crowding conditions lead to structural polymorphism of DNA sequences regulated by the state of hydration.

Introduction

Biomolecules have evolved to function within living cells, which contain a variety of biomolecules, including nucleic acids, proteins, and polysaccharides, as well as other soluble and insoluble components. These biomolecules occupy a significant fraction (20−40%) of the cellular volume.[1] The total concentration of the biomolecules can reach 400 g L^{-1}, leading to a crowded intracellular environment, which is optimized for biomolecular functions. Most biochemical studies of biomolecules, however, are performed under dilute conditions.[2] The effect of molecular crowding on the structure and stability of biomolecules has become a subject of increasing interest because it may clarify how biomolecules behave under cell-mimicking conditions.[3]

To date, there have been few systematic studies on the effect of molecular crowding on the stability of DNA structures.[4−6] We previously reported that the length of DNA and size of the cosolute are key factors determining the hybridization energy of DNA duplexes through Watson−Crick base pairs under molecular crowding conditions.[4] We also reported that most of the effects of molecular crowding on the stability of the DNA are due to changes in water activity and DNA hydration.[4] Spink and Chaires as well as and Goobes and Minsky demonstrated that DNA triplexes consisting of Hoogsteen base pairs are stabilized by molecular crowding. These groups also suggested that the thermodynamics of DNA duplexes and triplexes are regulated by DNA hydration.[7]

Understanding the effects of molecular crowding on not only duplexes and triplexes but also G-quadruplexes is important because there is growing interest in the potential roles of G-quadruplexes in biological systems.[8] G-quadruplexes are formed by intermolecular or intramolecular association of guanine-rich oligonuleotides with four Hoogsteen-paired coplanar guanines, a structure called a G-quartet (Figure 1a).[9] We

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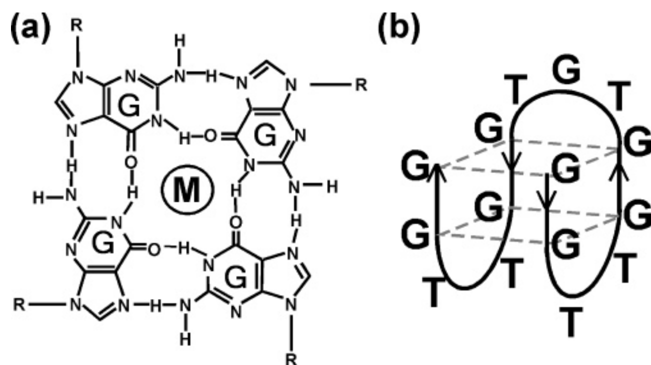

Figure 1. (a) Chemical structure of a G-quartet. M indicates a monovalent cation coordinated to the G-quartet. (b) Schematic structure of a thrombin DNA aptamer.

and another group have reported that molecular crowding causes a structural transition from an antiparallel to a parallel DNA G-quadruplex and from a DNA duplex to a DNA quadruplex.[10] Li et al. also reported that molecular crowding with poly(ethylene glycol) (PEG) induces dramatic changes in the quadruplex structure in the presence of K^+.[11] These previous results suggest that molecular crowding is one of the most important factors controlling the formation of G-quadruplex structures and, therefore, that it is a key factor in a variety of biological systems where guanine-rich sequences are important.

The thermodynamics of G-quadruplex formation under molecular crowding conditions remain unclear. Moreover, it is not fully understood how molecular crowding affects the structure and stability of DNA oligonucleotides. Therefore, systematic and quantitative studies on the effect of molecular crowding on the DNA structures are needed. In particular, the effects of molecular crowding not only on canonical B-form DNA duplexes containing Watson−Crick base pairs but also on noncanonical DNA structures, such as G-quadruplexes containing Hoogsteen base pairs, can clarify the differences in the thermodynamics governing the formation of DNA structures under dilute and molecular crowding conditions, and such studies establish how various DNA oligonucleotides form structures under cell-like conditions where water activity decreases and hydration is unfavorable.

Here, we systematically studied the effect of molecular crowding induced by neutral cosolutes on the thermodynamics of intramolecular antiparallel G-quadruplex formation with Hoogsteen base pairs and of intramolecular antiparallel hairpin-looped duplex (HP duplex) formation via Watson−Crick base pairs. We found that the antiparallel G-quadruplex is stabilized under molecular crowding conditions at various concentrations of PEG 200 (PEG with an average molecular weight [M_w] of 200) but that these conditions destabilize the HP duplex. Plots of stability ($\ln K_{obs}$) of the DNA structures versus water activity ($\ln a_w$) demonstrated that the $\ln K_{obs}$ for G-quadruplex formation decreases linearly as the $\ln a_w$ increases, whereas that for duplex formation increases linearly as the $\ln a_w$ increases. This suggested that the slope approximately equals the number of water molecules released or taken up during the formation of the DNA structures. Furthermore, on the basis of these results, we estimated for the first time that the number of water molecules released per nucleotide upon formation of an antiparallel G-quadruplex in the presence of K^+ and Na^+ are 4.5 $\pm$ 0.4 and 4.0 $\pm$ 0.1, respectively. Conversely, we estimated the number of water molecules taken up per nucleotide upon formation of the HP duplex in the presence of K^+ and Na^+ to be 3.4 $\pm$ 0.6 and 3.5 $\pm$ 0.4, respectively. Moreover, analysis of the effect of cosolute structure on the thermodynamics of antiparallel G-quadruplex and HP duplex formation showed that molecular crowding affects the thermodynamics of DNA structure formation by altering the hydration of the DNA. Specifically, a cosolute with fewer hydroxyl groups in the vicinal position leads to a larger number of water molecules released or taken up during the formation of the antiparallel G-quadruplex or the HP duplex, respectively. These results imply that, under molecular crowding conditions, noncanonical DNA structures, such as G-quadruplexes and triplexes consisting of Hoogsteen base pairs, are more favored than canonical DNA duplexes consisting of Watson−Crick base pairs. In this way, the structure of various DNA sequences can be regulated by the state of hydration.

Results and Discussion

Sequence Design and Structural Analysis. To estimate the thermodynamic parameters of a G-quadruplex, a DNA oligonucleotide must undergo a two-state transition between a single-stranded random coil and a G-quadruplex depending on the conditions.[12] We previously reported that guanine-rich sequences derived from *Oxytricha* nova and *Tetrahymena* telomere DNAs undergo a structural transition between antiparallel and parallel G-quadruplexes that depends on cosolute concentrations.[10a−c] In addition, human telomere DNAs fold into antiparallel and parallel G-quadruplexes in the presence of Na^+ and K^+, respectively.[13] This structural polymorphism of G-quadruplexes makes their thermodynamics difficult to analyze quantitatively. Thus, to perform the measurements, we needed a guanine-rich DNA that folds into a G-quadruplex structure via a two-state transition under various conditions. We tested a thrombin DNA aptamer d($G_2T_2G_2TGTG_2T_2G_2$) (Figure 1b)[14] because it has been reported to fold into an antiparallel G-quadruplex structure in the presence of various monovalent and divalent cations.[15] Figure S1a shows circular dichroism (CD) spectra of the thrombin DNA aptamer at 4 °C in the presence of K^+ or Na^+ and with or without 40 wt % of PEG 200. All CD spectra had large positive and negative peaks around 295 and 265 nm, respectively, indicating an antiparallel G-quadruplex structure under all of the conditions.[10c,16] In addition, the CD intensities at 295 nm in the presence of K^+ were changed little by PEG 200, whereas those in the presence of Na^+ were much higher with than without PEG 200. These results suggest that the

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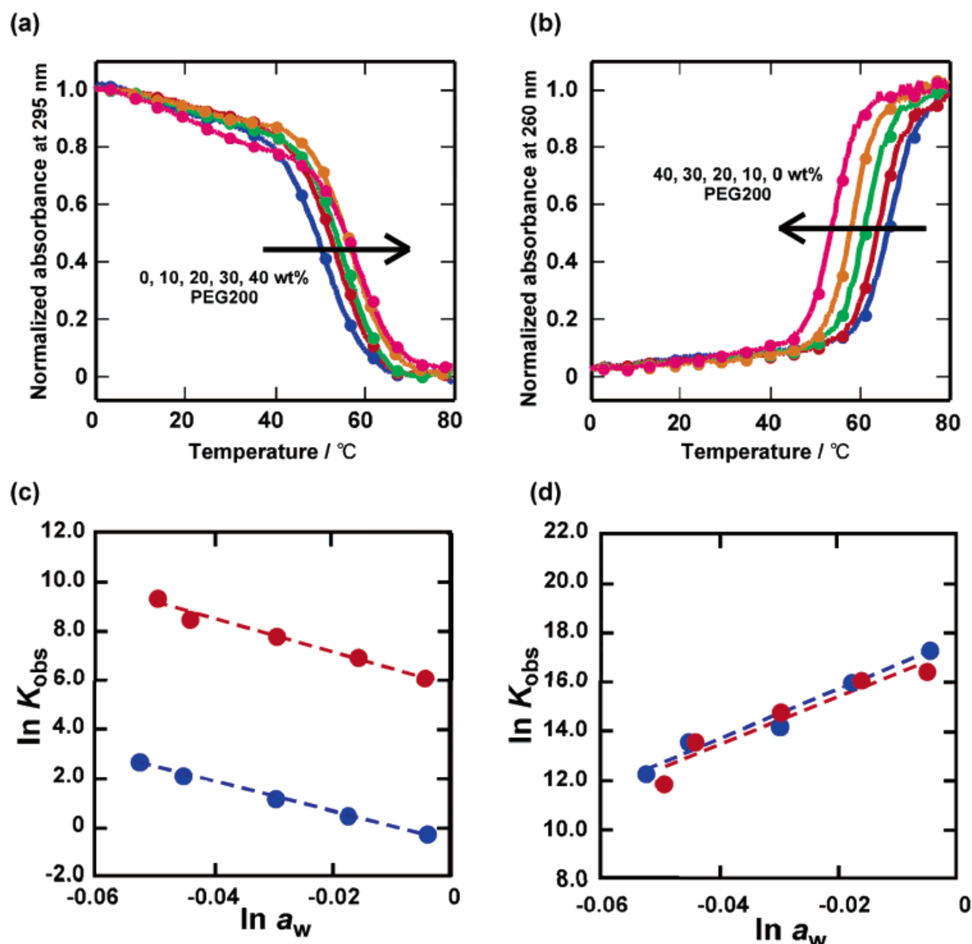

Figure 2. (a) Normalized UV melting curves for 5 μM G-quadruplex and (b) normalized UV melting curves for 5 μM HP duplex in a buffer of 100 mM KCl, 10 mM K_2HPO_4 (pH 7.0), and 1 mM K_2EDTA containing 0 wt % (blue), 10 wt % (red), 20 wt % (green), 30 wt % (orange), or 40 wt % (pink) PEG 200. (c) Plots of ln K_{obs} versus ln a_w for the formation of the G-quadruplex and (d) ln K_{obs} versus ln a_w for the formation of the HP duplex in buffers of 100 mM KCl, 10 mM K_2HPO_4 (pH 7.0), and 1 mM K_2EDTA (red) or 100 mM NaCl, 10 mM Na_2HPO_4 (pH 7.0), and 1 mM Na_2EDTA (blue) containing 0, 10, 20, 30, or 40 wt % PEG 200 at 25 °C. Melting of the antiparallel G-quadruplex and the HP duplex was assessed by UV absorbance at 295 and 260 nm, respectively.

antiparallel G-quadruplex is very stable in the presence of K^+ and that, in the presence of Na^+, the structure is stabilized by molecular crowding. Moreover, we performed native gel electrophoresis in the presence of K^+ or Na^+ and 0, 10, 20, 30, or 40 wt % of PEG 200 (Figure S1b). We found that the aptamer migrated faster than a single-stranded 15-mer DNA oligonucleotide under all conditions. A previous report showed that an intramolecular antiparallel G-quadruplex migrates faster than a single-stranded oligonucleotide of the same length, whereas an intermolecular parallel G-quadruplex migrates slower than the single-stranded oligonucleotide.[10c] Thus, the native gel electrophoresis indicates that the aptamer forms an antiparallel G-quadruplex under all conditions. The results of the CD analysis and native gel electrophoresis show that a uniform antiparallel G-quadruplex structure is formed under all of the tested conditions. We also used a 28-mer DNA, d(TCTTTCTCT-TCTTTTTAGAAGAGAAAGA) (loop region is underlined), that folds into an intramolecular HP duplex for comparison with the antiparallel G-quadruplex (CD spectra and native gel electrophoresis of the HP duplex with or without PEG 200 are shown in Figure S1c and S1d, respectively).

We next examined the thermal denaturation and renaturation of the antiparallel G-quadruplex in the presence of K^+ or Na^+ with or without 40 wt % of PEG 200 (Figure S2). The denaturation and renaturation, as assessed by measuring UV absorbance at 295 nm,[17] were identical under all conditions, indicating a two-state transition between a single strand and an antiparallel G-quadruplex. Therefore, we used this thrombin DNA aptamer and the 28-mer DNA to evaluate the effects of molecular crowding on thermodynamic stability of intramolecular G-quadruplexes with Hoogsteen base pairs and HP duplexes with Watson–Crick base pairs.

Thermodynamics of G-Quadruplex and Duplex Formation under Molecular Crowding Conditions. The thermodynamic parameters for the formation of antiparallel G-quadruplexes and HP duplexes were obtained from their thermal melting curves, which are measured by UV absorbance at 295 and 260 nm, respectively.[17] Figure 2a shows UV melting curves for the antiparallel G-quadruplex in the presence of K^+ and various amounts of PEG 200. Surprisingly, the melting temperature (T_m) for 5 μM of the antiparallel G-quadruplex increased from 54.1 to 58.7 °C as the PEG 200 concentration was increased from 0 to 40 wt %. In the presence of Na^+, the T_m for 5 μM DNA also increased from 24.1 to 36.6 °C as the PEG 200 concentration was increased from 0 to 40 wt % (Figure S3a). These results demonstrate that the G-quadruplex structure

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Table 1. The Thermodynamic Parameters and Water Activity for the Antiparallel G-Quadruplex and HP Duplex Formation with Various Amounts of PEG 200[a]

cosolute	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1})	$\Delta G°_{25}$ (kcal mol^{-1})	$T_m{}^b$ (°C)	ln a_w (×10^2)
		G-quadruplex			
none	−42.0 ± 0.8	−38.5 ± 0.8	−3.5 ± 0.1	54.1	−0.42
10 wt % PEG 200	−45.2 ± 0.5	−41.2 ± 0.4	−4.0 ± 0.1	55.0	−1.55
20 wt % PEG 200	−47.8 ± 2.1	−43.3 ± 1.9	−4.5 ± 0.2	56.4	−2.94
30 wt % PEG 200	−50.9 ± 1.9	−46.0 ± 1.6	−5.0 ± 0.3	59.4	−4.40
40 wt % PEG 200	−53.0 ± 1.5	−47.5 ± 1.4	−5.5 ± 0.2	58.7	−4.95
		HP duplex			
none	−81.5 ± 0.8	−71.7 ± 0.7	−9.8 ± 0.2	66.4	−0.42
10 wt % PEG 200	−82.3 ± 1.7	−72.9 ± 1.6	−9.5 ± 0.2	63.6	−1.55
20 wt % PEG 200	−80.0 ± 1.6	−71.2 ± 1.6	−8.7 ± 0.3	61.0	−2.94
30 wt % PEG 200	−78.9 ± 0.3	−70.8 ± 0.4	−8.1 ± 0.5	58.1	−4.40
40 wt % PEG 200	−75.8 ± 0.9	−68.9 ± 0.7	−6.9 ± 0.3	54.3	−4.95

[a] All experiments were carried out in a buffer containing 100 mM KCl, 10 mM K_2HPO_4 (pH 7.0), 1 mM K_2EDTA, and various concentrations of PEG 200. Thermodynamic parameters are the average values obtained from melting curves with at least four different concentrations of DNA oligonucleotides. [b] The melting temperature was determined at a strand concentration of 5 μM.

is stabilized by molecular crowding conditions. Previously, it was reported that, under dilute conditions, G-quadruplexes are more stable in the presence of K^+ than Na^+ because of the optimal fit of K^+ in the coordination sites (O6 of guanine bases) formed by G-quartets.[18] In support of our CD spectral results (Figure S1a), the T_m measurements indicate that the antiparallel G-quadruplex is more stable in the presence of K^+ than Na^+ under both dilute and molecular crowding conditions.

In contrast to the G-quadruplex, the T_m for 5 μM of the HP duplex in the presence of K^+ decreased from 66.4 to 54.3 °C as the PEG 200 concentration was increased from 0 to 40 wt % (Figure 2b). The T_m of the HP duplex also decreased from 67.0 to 55.5 °C as the PEG 200 concentration was increased from 0 to 40 wt % in the presence of Na^+ (Figure S3b). The destabilization of the duplex by molecular crowding agrees with previous reports showing destabilization of the poly(dT)/poly(dA) duplex by ethylene glycol[7] and of 8-mer, 17-mer, and 30-mer duplexes by ethylene glycol, PEG 200, or PEG 1000.[4] In addition, the T_m values for the HP duplex in the presence of K^+ or Na^+ demonstrated that, as under dilute conditions,[19] the thermodynamic stabilities of the duplex under molecular crowding conditions are nearly the same with the different monovalent cations.

Furthermore, we estimated the thermodynamic parameters ($\Delta H°$, $T\Delta S°$, and $\Delta G°$) for the antiparallel G-quadruplex and HP duplex formation in the presence of various concentrations of PEG 200. Table 1 shows the values of $\Delta H°$, $T\Delta S°$, and $\Delta G°$ at 25 °C ($\Delta G°_{25}$) for the formation of the structures as well as the water activities in the presence of 100 mM K^+ and various amounts of PEG 200. When the PEG 200 concentration was increased from 0 to 40 wt %, the values of $\Delta H°$, $T\Delta S°$, and $\Delta G°_{25}$ of the antiparallel G-quadruplex decreased from −42.0 to −53.0 kcal mol^{-1}, from −38.5 to −47.5 kcal mol^{-1}, and from −3.5 to −5.5 kcal mol^{-1}, respectively. These changes indicate that promotion of G-quadruplex formation by PEG 200 is enhanced by a favorable enthalpic contribution that exceeds an unfavorable entropic contribution. On the other hand, the values of $\Delta H°$, $T\Delta S°$, and $\Delta G°_{25}$ for formation of the HP duplex increased from −81.5 to −75.8 kcal mol^{-1}, from −71.7 to −68.9 kcal mol^{-1}, and from −9.8 to −6.9 kcal mol^{-1}, respectively, with the same change of PEG 200 concentration. These changes indicate that destabilization of the HP duplex by PEG 200 is due to an unfavorable enthalpic contribution that exceeds a favorable entropic contribution.

Effect of Water Molecules on the Stability of the Antiparallel G-Quadruplex and the HP Duplex. Next, we investigated how water molecules affect the thermodynamic stability of the antiparallel G-quadruplex and the HP duplex. Formation of an intramolecular structure by a DNA strand in an aqueous solution containing a cosolute (i.e., PEG 200) and a cation (i.e., K^+ or Na^+) can be represented as follows:

$$A_{ss} \rightleftarrows A_f + \Delta n_w H_2O + \Delta n_{CS} CS + \Delta n_{M^+} M^+ \quad (1)$$

where A_{ss} and A_f represent single-stranded and formed DNA structures, respectively; CS is the cosolute; M^+ is a metal cation; and Δn_w, Δn_{cs}, and Δn_{M^+} are the numbers of water, cosolute, and cation released upon formation of the structure, respectively.[4,7b,20] The observed equilibrium constant (K_{obs}) for the formation of the structure is thus given as

$$K_0 = K_{obs} a_w^{\Delta n_w} a_{CS}^{\Delta n_{CS}} a_{M^+}^{\Delta n_{M+}} \quad (2)$$

where K_0 is the true thermodynamic equilibrium constant, and a_w, a_{cs}, and a_{M^+} are the activities of water, cosolute, and cation, respectively. At constant temperature and pressure, the derivative of ln K_{obs} by ln a_w is represented by eq 3, which contains the terms for the number of bound molecules:

$$\frac{\partial \ln K_{obs}}{\partial \ln a_w} = -\left[\Delta n_w + \Delta n_{CS}\left(\frac{\partial \ln a_{CS}}{\partial \ln a_w}\right) + \Delta n_{M^+}\left(\frac{\partial \ln a_{M^+}}{\partial \ln a_w}\right)\right] \quad (3)$$

In this study, Δn_w, Δn_{CS}, and Δn_{M^+} are the numbers of water, cosolute (PEG 200), and K^+ or Na^+, respectively, released upon formation of the antiparallel G-quadruplex or the HP duplex.

Figure 2c shows the plots of ln K_{obs} for antiparallel G-quadruplex formation at 25 °C versus ln a_w as determined by osmotic pressure measurements at 25 °C.[4] The plot reveals that, in the presence of K^+ or Na^+, the stability of the antiparallel G-quadruplex (ln K_{obs}) decreases linearly with the increase in ln a_w. In contrast to the antiparallel G-quadruplex, the plots of ln K_{obs} for the HP duplex at 25 °C versus ln a_w showed that the

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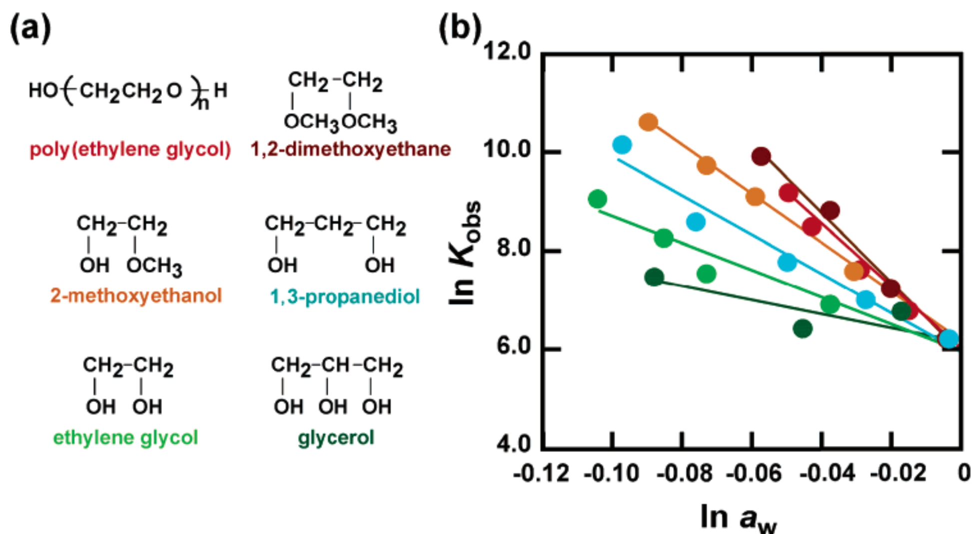

Figure 3. (a) Chemical structures of the cosolutes used in this study. (b) Plots of ln K_{obs} versus ln a_w for the G-quadruplex in buffers of 100 mM KCl, 10 mM K_2HPO_4 (pH 7.0), and 1 mM K_2EDTA containing various concentrations of PEG 200 (red), 1,2-dimethoxyethane (brown), 2-methoxyethanol (orange), 1,3-propanediol (blue), ethylene glycol (bright green), or glycerol (dark green) at 25 °C.

ln K_{obs} increased linearly with the increase in ln a_w (Figure 2d). Although the slopes of the plots include the two variable terms related to cosolute and cation binding, the plots in Figure 2c and 2d are linear, suggesting that the variable terms are insignificant, and thus, the slope is approximately equal to the constant term $-\Delta n_w$.[4,21] The slopes of ln K_{obs} versus ln a_w for the G-quadruplex in the presence of K^+ or Na^+ were estimated to be -67.0 ± 5.2 and -60.3 ± 1.8, respectively. These values correspond to release of 4.5 ± 0.4 and 4.0 ± 0.1 water molecules per nucleotide upon formation of an antiparallel G-quadruplex in the presence of K^+ and Na^+, respectively. Conversely, the slopes of the HP duplex formation in the presence of K^+ (95.6 $\pm$ 15.7) or Na^+ (99.1 $\pm$ 9.9) indicate 3.4 ± 0.6 and 3.5 ± 0.4 water molecules taken up per nucleotide upon formation of an HP duplex in the presence of K^+ or Na^+, respectively. Notably, these results demonstrated dehydration and hydration during the formation of an antiparallel G-quadruplex and an HP duplex, respectively. In addition, the number of water molecules released or taken up during the formation of these structures did not depend on the cation present.

Origin of Water Molecules Released and Taken Up During the Formation of the DNA Structures. To further confirm the importance of water molecules in the thermodynamic stability of the DNA structures and to study the origin of water molecules released or taken up during their formation, we examined the effects of various low-molecular weight cosolutes with different structures (Figure 3a). Figure 3b shows the plot of ln K_{obs} at 25 °C versus ln a_w for the antiparallel G-quadruplex in the presence of K^+ and various concentrations of ethylene glycol (M_w = 62), glycerol (M_w = 92), 1,3-propanediol (M_w = 76), 2-methoxyethanol (M_w = 76), 1,2-dimethoxyethane (M_w = 90), or PEG 200. The plot indicates that the stability of the G-quadruplex (ln K_{obs}) decreased linearly with the increase in ln a_w for all of the tested cosolutes. The slope of 1,2-dimethoxyethane is close to that of PEG 200. Moreover, the absolute values of the slope decrease in the following order: PEG 200 ≈ 1,2-dimethoxyethane > 2-methoxyethanol > 1,3-propanediol > ethylene glycol > glycerol. The numbers of hydroxyl groups in the vicinal position in PEG 200, 1,2-dimethoxyethane, 2-methoxyethanol, 1,3-propanediol, ethylene glycol, and glycerol are 0, 0, 1, 1, 2, and 3, respectively. In the case of the duplex, PEG 200 and 1,2-dimethoxyethane most significantly destabilized the duplex, whereas ethylene glycol and glycerol only slightly destabilized the duplex.[4] Thus, the order of the absolute value of the slope is the same as that observed for the G-quadruplex, although, in this case, the cosolutes had the opposite effect (destabilization) on the structure. These observations demonstrate that a cosolute with fewer hydroxyl groups in the vicinal position causes more water molecules to be released during formation of the antiparallel G-quadruplex or to be taken up during formation of the duplex. Therefore, it is possible that the cosolutes used here affect the thermodynamics for the formation of an antiparallel G-quadruplex or an HP duplex by regulating the hydration of the DNA molecule.

A previous study showed that molecular crowding by putrescine, a positively charged polymer, destabilizes an antiparallel G-quadruplex of d($G_4T_4G_4$) due to direct electrostatic interactions with DNA strands.[10a] This is not the case for the PEG used here because we and other researchers have shown that a direct interaction between PEG and DNA strands is thermodynamically unfavorable.[10a,21] Thus, it appears that indirect interactions with cosolutes containing fewer hydroxyl groups, such as PEG, affect the hydration of the DNA structures. On the other hand, direct interactions can occur between DNA and cosolutes with more hydroxyl groups, such as glycerol. The cosolutes with more hydroxyl groups can bind to DNA molecules in single-stranded and structured states. During DNA structure formation, cosolutes may be released or taken up along with the uptake or release of water molecules, respectively. Therefore, it is possible that the solvation of nucleotides by cosolutes with more hydroxyl groups eliminates the uptake or release of water molecules. In these ways, the changes in hydration of the DNA structures should lead to the enthalpy and entropy changes associated with antiparallel G-quadruplex and HP duplex formation under molecular crowding conditions (Table 1).

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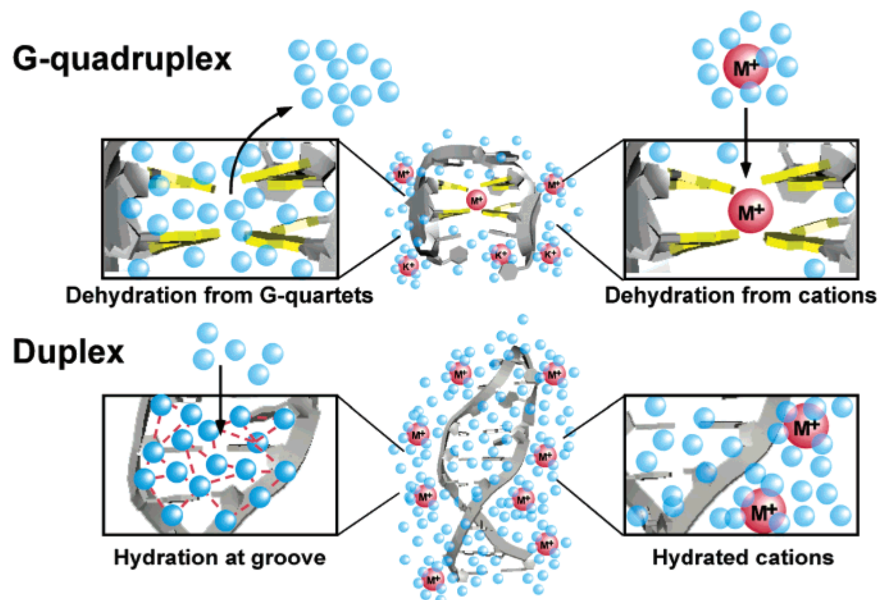

Figure 4. Schematic illustrations of the dehydration of the G-quadruplex and the hydration of the HP duplex. Blue and red circles indicate water molecule and cations, respectively.

On the basis of these findings, we developed a model, shown schematically in Figure 4, for the dehydration during G-quadruplex formation and the hydration during duplex formation. In the case of the G-quadruplex, the water molecules are released from the guanine bases because the four atoms (N1, N2, O6, and N7) of guanine are occupied by the cyclic Hoogsteen base pairs.[14] Moreover, the water molecules should be released from the cations because their full dehydration is required for cation−G-quartet binding.[22] Kankia and Marky reported that the dehydration of both guanine bases and cations controls the thermodynamics of the antiparallel G-quadruplex.[15] Hud et al. also demonstrated that the preferred coordination of K^+ over Na^+ is driven by the greater energetic cost of Na^+ dehydration compared to K^+ dehydration,[23] which can explain why the number of water molecules released during the formation of the antiparallel G-quadruplex is slightly higher in the presence of K^+ than Na^+ (-67.0 ± 5.2 and -60.3 ± 1.8, respectively; Figure 2c). The dehydration of the G-quadruplex and the cations leads to stabilization of the structure under molecular crowding conditions because the activity of water molecules and the number of water molecules around the DNA strands decrease. In contrast, duplex formation requires hydration because Watson−Crick base pairs have more hydration sites than cyclic Hoogsteen base pairs and because dehydration of cations is not required due to their diffuse binding to the HP duplex.[24] As a result, the numbers of water molecules taken up during HP duplex formation in the presence of K^+ (95.6 ± 15.7) or Na^+ (99.1 ± 9.9) is not significantly different (Figure 2d). These results demonstrated that the number of water molecules released or taken up during the formation of the DNA structures is a key factor in the thermodynamics under molecular crowding conditions, although further investigation, such as measurements of the changes in the molar volume and compressibility, are needed.

Importance of G-Quadruplex Stabilization under Molecular Crowding Conditions. In this study, we demonstrated that molecular crowding conditions lead to stabilization of the G-quadruplex and destabilization of the duplex (Figure 2 and Table 1). Moreover, the thermodynamic parameters for the formation of the DNA structures under dilute and molecular crowding conditions indicated that hydroxyl groups in the vicinal position of the cosolutes can regulate the thermodynamics of DNA structure formation (Figure 3). It is noteworthy that water molecules along the grooves of a DNA duplex form hydrogen bonds to the nucleotides, phosphate groups, and sugar rings, creating a specific hydration pattern in the grooves of a DNA duplex.[25] Therefore, these water molecules contribute to the total number of water molecules taken up or released upon formation of DNA structures. For example, the water molecules involved in the specific hydration pattern should be taken up upon duplex formation through Watson−Crick base pairs, whereas they should be released upon triplex formation, in which a third strand hybridizes to a Watson−Crick duplex through Hoogsteen base pairs. In fact, it was previously reported that a DNA triplex consisting of Hoogsteen base pairs is stabilized as the water activity decreases.[5−7] Moreover, a theoretical study of hydration by a parallel-stranded duplex consisting of Hoogsteen base pairs and an antiparallel-stranded duplex consisting of Watson−Crick base pairs indicated that the hydration site of the parallel-stranded duplex is much smaller than that of the antiparallel-stranded duplex.[26] Thus, it appears that molecular crowding conditions, wherein water activity decreases and hydration is unfavorable, stabilize DNA structures containing Hoogsteen base pairs and destabilize those containing Watson−Crick base pairs.

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Further studies on the thermodynamic stability of various DNA structures under molecular crowding conditions with various cosolutes are needed, but antiparallel G-quadruplex structures with human telomeric DNAs are stabilized by molecular crowding conditions (our unpublished observations), and both intramolecular and intermolecular duplexes are destabilized by molecular crowding.[4] These results imply that noncanonical DNA structures, such as G-quadruplexes and triplexes containing Hoogsteen base pairs, can be favored over canonical DNA duplexes containing Watson−Crick base pairs depending on the surrounding conditions and the DNA sequence. This leads to structural polymorphism of various DNA sequences that is regulated by the state of hydration.

Experimental Section

Materials. All oligodeoxynucleotides used in these studies were high performance liquid chromatography grade and were purchased from Hokkaido System Science (Sapporo, Japan). Single-strand concentrations of the DNA oligonucleotides were determined by measuring the absorbance at 260 nm at high temperature using a Shimadzu 1700 spectrophotometer (Shimadzu, Kyoto, Japan) connected to a thermoprogrammer. Single-strand extinction coefficients were calculated from mononucleotide and dinucleotide data using the nearest-neighbor approximation.[27]

Water Activity Measurements. The water activity was determined by the osmotic stressing method via vapor phase osmometry using a model 5520XR pressure osmometer (Wescor, Utah, USA) or by freezing point depression osmometry using a Typ Dig. L osmometer (KNAUER, Berlin, Germany), with the assumption that the cosolutes do not directly interact with DNAs.[4,7a,20]

CD Measurements. CD experiments utilizing a JASCO J-820 spectropolarimeter (JASCO, Hachioji, Japan) were measured at 4 °C in a 0.1 cm path length cuvette for 50 μM total strand concentration of DNA in buffers of 100 mM KCl or NaCl, 10 mM K_2HPO_4 or Na_2HPO_4 (pH 7.0), and 1 mM K_2EDTA or Na_2EDTA containing various concentrations of PEG 200. The CD spectra were obtained by taking the average of at least three scan made from 200 to 350 nm. The temperature of the cell holder was regulated by a JASCO PTC-348 temperature controller, and the cuvette-holding chamber was flushed with a constant stream of dry N_2 gas to avoid condensation of water on the cuvette exterior. Before the measurement, the sample was heated to 80 °C, gently cooled at a rate of 2 °C min^{-1}, and incubated at 4 °C for 1 h.

Gel Electrophoresis. Native gel electrophoresis was carried out on nondenaturing gels containing 20% polyacrylamide. Ice-cold loading buffer (2 μL) was mixed with 2 μL of 5 μM DNA sample in buffers of 100 mM KCl or NaCl, 10 mM K_2HPO_4 or Na_2HPO_4 (pH 7.0), and 1 mM K_2EDTA or Na_2EDTA containing various concentrations of PEG 200. A 2 μL aliquot of the mixed solution was loaded and analyzed by electrophoresis at 5 V cm^{-1} for 6 h at 4 °C. Gels were stained using GelStar and imaged using a FLA-5100 (Fuji Photo Film Co., Ltd., Tokyo, Japan). Before the measurement, the sample was heated to 80 °C, gently cooled at a rate of 2 °C min^{-1}, and incubated at 4 °C for 1 h.

Thermodynamic Analysis. The UV absorbance was measured with a Shimadzu 1700 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a temperature controller. Melting curves of G-quadruplexes and HP duplex were obtained by measuring the UV absorbance at 295 and 260 nm, respectively, in buffers containing 100 mM KCl or NaCl, 10 mM K_2HPO_4 or Na_2HPO_4 (pH 7.0), and 1 mM K_2EDTA or Na_2EDTA supplemented with various concentrations of cosolutes. The T_m values for 5 μM G-quadruplexes and HP duplex were obtained from the UV melting curves as described previously.[27] The heating rate was 0.5 °C min^{-1} for both the G-quadruplex and the HP duplex because the shape of the melting curve and T_m were unaffected by heating rates between 0.1 and 0.5 °C min^{-1}. The thermodynamic parameters were calculated from the fit of the melting curves (with at least four different concentrations of DNA oligonucleotides) to a theoretical equation for an intramolecular association as described previously.[27] The thermodynamic parameters listed in Table 1 are the average values obtained from each melting curve and curve fitting analysis. Before the measurement, the sample was heated to 80 °C, gently cooled at a rate of 2 °C min^{-1}, and incubated at 0 °C for 1 h.

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Supporting Information Available: CD spectra and native gel electrophoresis of the antiparallel G-quadruplex and HP duplex, the thermal denaturation and renaturation curves of the G-quadruplex, and normalized UV melting curves for the antiparallel G-quadruplex and the HP duplex in the presence of Na^+ with various concentrations of PEG200. This material is available free of charge via the Internet at http://pubs.acs.org.

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