Molecular Crowding Regulates the Structural Switch of the DNA G-Quadruplex†

Daisuke Miyoshi,‡ Akihiro Nakao,‡ and Naoki Sugimoto*,‡,§

Department of Chemistry, Faculty of Science and Engineering, and High Technology Research Center, Konan University, 8-9-1 Okamoto, Higashinada-ku, Kobe 658-8501, Japan

Received June 11, 2002

ABSTRACT: Almost all biochemical reactions in vitro have been investigated through numerous experiments conducted in dilute solutions containing low concentrations of solutes. However, biomacromolecules such as nucleic acids, proteins, and polysaccharides are designed to function and/or form their native structures in a living cell containing high concentrations of biomacromolecules, substrates, cofactors, salts, and so on. In the present study, we have demonstrated quantitatively the effect of molecular crowding on structures and stabilities of the G-quadruplex of d($G_4T_4G_4$). Molecular crowding with poly(ethylene glycol) (PEG) induced a structural transition from the antiparallel to the parallel G-quadruplex of d($G_4T_4G_4$), while molecular crowding with polycations did not alter the structure of the antiparallel G-quadruplex. The binding constants of putrescine, one of the polycations, for d($G_4T_4G_4$) in the absence and presence of Na^+ are calculated to be 277 and 2.5 M^{-1}, respectively. This indicates that the polycations coordinate to d($G_4T_4G_4$) with electrostatic interactions. The thermodynamic parameters of the antiparallel G-quadruplex formation under the crowding and noncrowding conditions induced by putrescine were also estimated. The stability of the antiparallel G-quadruplex decreased ($-\Delta G°_{25}$ decreased from 28 to 22 kcal mol^{-1}) with molecular crowding by putrescine. Also, enthalpy and entropy changes in the structural formation under crowding and noncrowding conditions clearly showed that destabilization was entropy-driven. These quantitative parameters indicated that both the volume excluded by PEG and chemical interactions such as electrostatic interaction with solute polycations are critical for determining how molecular crowding affects the structure and stability of highly ordered DNA structures.

In a living cell, biomacromolecules such as a nucleic acid, protein, and polysaccharide together with other soluble and insoluble components occupy 30−40% of the cellular volume, and their total concentration is often 400 g/L (*1*, *2*). Therefore, biochemical reactions in vivo progress under crowding conditions containing high concentrations of biomacromolecules. Most studies of the biochemical reactions in vitro, however, have been performed in solutions containing low concentrations of biomacromolecules (less than 1 mg/mL). While the effect of molecular crowding on biochemical reactions has been investigated theoretically (*3*−*7*), few systematic experimental studies are available. It has previously been reported that molecular crowding is a critical factor in determining the structure, stability, and activity of a protein (*8*−*11*). In addition, to understand physiology and metabolism in vivo, elucidation of the effect of molecular crowding on DNA structures and their stabilities is currently of great interest. However, the effect of the molecular crowding on the G-quadruplex[1] structure, which is one of the highly ordered structures of a nucleic acid, has not been demonstrated until now, although it has been shown that molecular crowding affects the thermodynamics of a triple helix DNA (*12*, *13*).

An important highly ordered structure of a nucleic acid is the G-quadruplex that is formed by guanine-rich oligonucleotides in the presence of certain cations such as Na^+ and K^+ (*14*). The G-quadruplex is stabilized by G-quartets, which are formed by the cyclic hydrogen bonds of four guanine bases in a coplanar arrangement (Figure 1a) (*14*). Although direct evidence of G-quadruplex formation and function in vivo is still lacking, there is currently great interest in the G-quadruplex structure because several guanine-rich sequences such as telomeres (*15*), the immunoglobin switch region (*16*), fragile X (*17*), and *c-myc* promoter (*18*) exist in the genome. NMR and X-ray diffraction studies as well as gel and spectroscopic techniques have shown that G-quadruplexes form intramolecular, dimer, tetramer, and highly ordered associations depending on their sequences (*19*, *20*). These studies have shown that the guanine-rich strands associate in parallel or antiparallel orientations and thus generate structural polymorphism. Switching between antiparallel and parallel G-quadruplexes has been proposed as possibly playing an important role in promoting chromosome association during meiosis (*20*, *21*), but the structure of the G-quadruplex under crowding conditions in a living cell is not yet clear.

In the present study, the effect of molecular crowding induced by polycations and neutral polymers on the telomeric

† This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan, to N.S.

* To whom correspondence should be addressed. Phone: +81-78-435-2497. Fax: +81-78-435-2539. E-mail: sugimoto@konan-u.ac.jp.

‡ Department of Chemistry, Faculty of Science and Engineering.

§ High Technology Research Center.

[1] Abbreviations: G-quadruplex, guanine quadruplex; G-quartet, guanine quartet; PEG, poly(ethylene glycol); CPG, controlled pore glass; HPLC, high-performance liquid chromatography; TEAA, triethylamine acetate; CD, circular dichroism; MES, 2-morpholinoethanesulfonic acid.

10.1021/bi020412f CCC: $22.00
Published on Web 11/20/2002

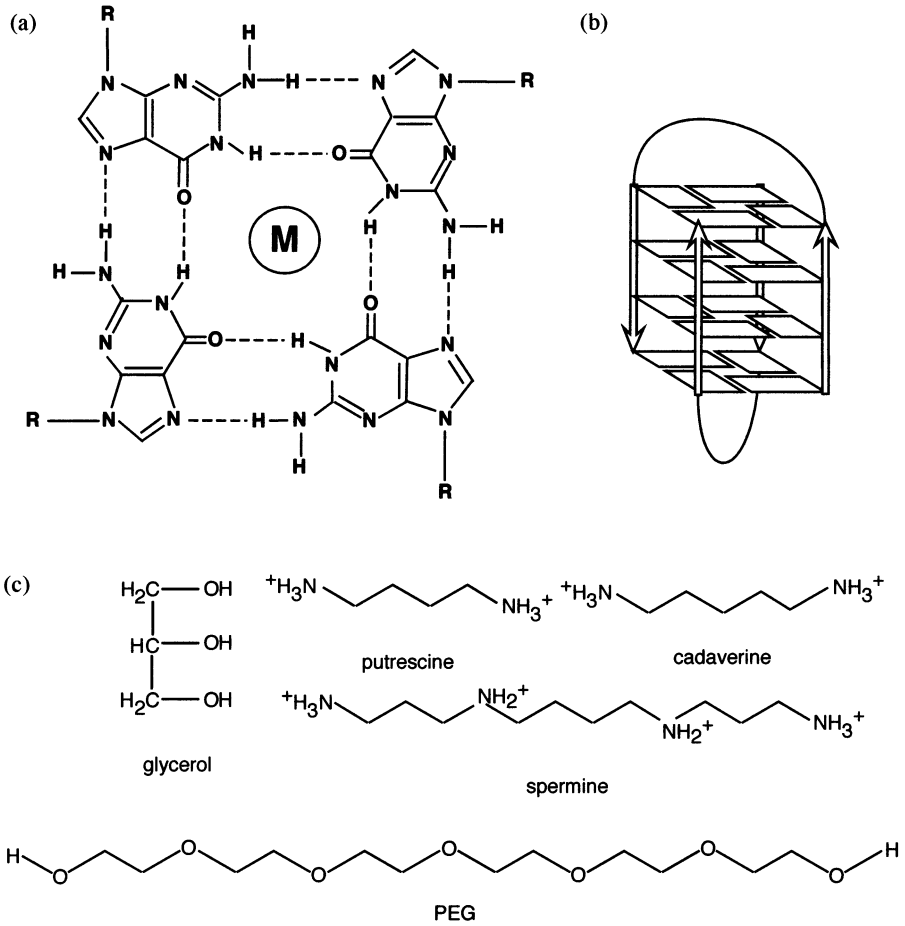

FIGURE 1: (a) Chemical structure of the G-quartet that is formed by Hoogsteen base pairs of four guanine bases. M and R indicate a cation and sugar, respectively. The dashed line indicates the Hoogsteen hydrogen bonds between guanine bases. (b) Schematic representation of the NMR structure of the d($G_4T_4G_4$) antiparallel G-quadruplex in the presence of Na^+. Arrows and squares indicate the strand directions and the planes of the G-quartet, respectively. Thymine bases are omitted for clarity. (c) Chemical structures of solutes used in this study.

repeat DNA of *Oxytricha nova*, d($G_4T_4G_4$), was investigated systematically and quantitatively. Although it has been reported that the structure of d($G_4T_4G_4$) in a dilute solution with Na^+ is the antiparallel G-quadruplex (*23*), as shown in Figure 1b, we found that d($G_4T_4G_4$) forms a parallel G-quadruplex under crowding conditions with 2 M PEG but not with 2 M putrescine in the presence of 100 mM NaCl. This indicates that molecular crowding plays a critical role in formation of the G-quadruplex structure of telomere DNA and therefore may regulate many biological processes concerned with guanine-rich sequences in vivo. Furthermore, the quantitative parameters of the G-quadruplex structure show that excluded volume, volume occupied by the solute that is unavailable to other solution components, and chemical interaction such as electrostatic interaction between DNA and polycations play a critical role in determining the structure and stability of the G-quadruplex under crowding conditions. This is the first report of the structural switch between the antiparallel and parallel G-quadruplex being regulated by molecular crowding.

MATERIALS AND METHODS

Materials. All of the oligodeoxynucleotides were chemically synthesized on a solid support by the phosphoramidite method as described previously (*24*). The synthesized DNA oligonucleotides were removed from the CPG column by treatment with 25% concentrated ammonia at 55 °C for 8 h. After being dried in a vacuum, the DNA oligonucleotides were passed through a Poly-Pak cartridge (Gren Research Co., Ltd.) to remove the dimethoxytrityl groups. After deblocking, the final purities of the DNA oligonucleotides were confirmed to be greater than 98% by HPLC on Wakosil-II 5C18RS cartridges with a linear gradient of 0−50% methanol/water containing 0.1 M TEAA (pH 7.0). These DNA oligonucleotides were desalted with a C-18 Sep-Pack cartridge before use.

Single-strand concentrations of the DNA oligonucleotides were determined by measuring the absorbance at 260 nm at high temperature using an Hitachi U-3210 spectrophotometer connected to an Hitachi SPR-10 thermoprogrammer. Single-strand extinction coefficients were calculated from mononucleotide and dinucleotide data using the nearest-neighbor approximation (*24*).

Solutes used here to induce molecular crowding, putrescine (MW 88.15), cadaverine (MW 102.18), spermine (MW 202.34), glycerol (MW 92.09), and PEG (MW 300 (average)), were of reagent grade quality purchased from Wako Pure Chemical Co., Ltd. (Japan) and used without further purification. Chemical structures of these solutes are shown in Figure 1c.

Structural Analysis. Recent conformational analysis of G-quadruplex structures revealed that the CD spectra of an antiparallel G-quadruplex structure had a positive peak near 295 nm and a negative peak near 265 nm, while a parallel

G-quadruplex structure had positive and negative peaks near 260 and 240 nm, respectively (*25*, *26*). With this information, the structural type of a G-quadruplex can be determined by a CD measurement. The CD spectra of DNA oligonucleotides were measured for 50 μM DNA total concentration using a J-820 spectropolarimeter (JASCO Co., Ltd., Japan) with a 0.1 cm path-length quartz cell at 5 °C and interfaced to a Dell OptiPlex Gxi computer. The CD spectrum was obtained by taking the average of at least three scans made at 0.1 nm intervals from 200 to 350 nm. Before CD spectroscopy, the DNA sample was heated to 90 °C, gently cooled at a rate of 3 °C/min, and incubated at 5 °C for several hours. 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 water condensation on the cuvette exterior.

Titration Experiments. The relationship between solute concentration and the structure of d($G_4T_4G_4$) was investigated with titration experiments using PEG and putrescine. The structure of d($G_4T_4G_4$) at each titration point was measured using CD after thermal treatment, as described above. For example, 50 μM d($G_4T_4G_4$) in a buffer containing 100 mM NaCl, 2 M PEG, and 50 mM MES (pH 6.1) was added to 50 μM d($G_4T_4G_4$) in 100 mM NaCl and 50 mM MES (pH 6.1). Because two and four strands form antiparallel and parallel G-quadruplexes, respectively, there is a possibility that the effect of the concentration of d($G_4T_4G_4$) on the G-quadruplexes differs (*20*). Therefore, all CD spectra were measured for 50 μM d($G_4T_4G_4$) in a 0.1 cm path-length cuvette in a buffer containing 50 mM MES (pH 6.1), 100 mM NaCl, and appropriate PEG or putrescine concentration (from 0 mM to 2 M) at 5 °C. Before the CD measurement, all of the samples were thermally treated as described above.

Determination of Thermodynamic Parameters for Structural Formations. All melting curves of antiparallel and parallel G-quadruplexes were measured with the CD intensity at 295 and 260 nm, respectively. Samples were heated from 0 to 90 °C at a rate of 1.0 °C/min. Before the CD spectroscopy, all of the samples were thermally treated as described above. Because the antiparallel G-quadruplex structure of d($G_4T_4G_4$) is formed with two single strands (*27*), the helix−coil transition of d($G_4T_4G_4$) is represented as

$$2S \rightleftarrows Q \quad (1)$$

Here S and Q are single-strand DNA and the antiparallel G-quadruplex, respectively [if the structure of d($G_4T_4G_4$) is the antiparallel G-quadruplex]. The melting curves of the antiparallel G-quadruplex were fitted to a curve to obtain the three thermodynamic parameters, the enthalpy ($\Delta H°$), entropy ($\Delta S°$), and free energy changes ($\Delta G°_{25}$) at 25 °C, for the formation of the antiparallel G-quadruplex with the theoretical equation (*28*, *29*):

$$K(T) = \alpha/[2C_t(1-\alpha)^2] = \exp[(-\Delta H° + T\Delta S°)/RT] \quad (2)$$

Here α is the fraction of the double-stranded antiparallel G-quadruplex. The free energy changes for antiparallel G-quadruplex formation at 25 °C were then calculated:

$$\Delta G°_{25} = \Delta H° - T\Delta S° \quad (3)$$

Here T is the temperature (298.15 K). The thermodynamic parameters of a parallel-stranded G-quadruplex cannot be obtained because the stoichiometry of the single strand and parallel G-quadruplex has not been determined and the helix−coil transition of the parallel-stranded G-quadruplex of d($G_4T_4G_4$) has not been confirmed.

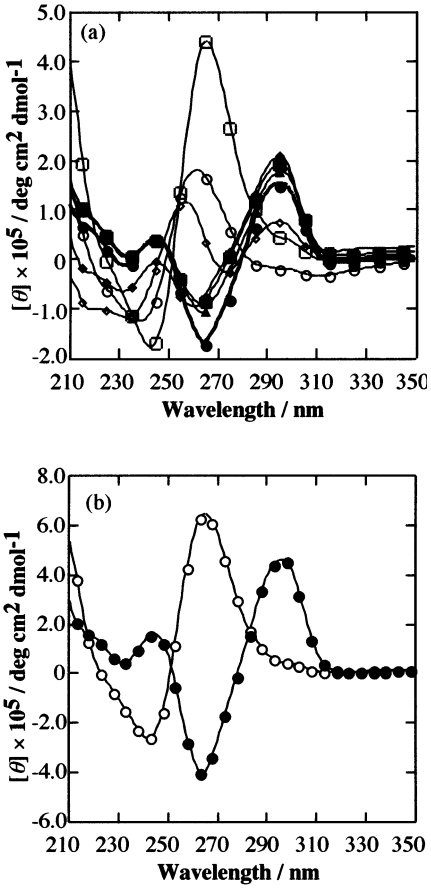

FIGURE 2: (a) CD spectra of 50 μM d($G_4T_4G_4$) at 5 °C in buffers containing 100 mM NaCl, 50 mM MES in the absence (shaded circle) and presence of 50 mM $CaCl_2$ (unshaded circle), 2 M putrescine (shaded square), 2 M cadaverine (shaded diamond), 1 M spermine (shaded triangle), 2 M PEG (unshaded square), or 2 M glycerol (unshaded diamond). (b) CD spectra of 50 μM d(G_4T_4)$_3G_4$ at 5 °C in buffers containing 100 mM NaCl and 50 mM MES in the absence (shaded circle) and presence of 2 M PEG (unshaded circle).

RESULTS AND DISCUSSION

Effect of Molecular Crowding Induced by Polycations, Glycerol, or PEG on the Structure of d($G_4T_4G_4$). Figure 2 shows the CD spectra of 50 μM d($G_4T_4G_4$) in a buffer containing 100 mM NaCl and 50 mM MES (pH 6.1) plus 2 M putrescine, 2 M cadaverine, 1 M spermine, 2 M glycerol, 2 M PEG, or 50 mM $CaCl_2$, together with a spectrum in a buffer containing 100 mM NaCl and 50 mM MES (pH 6.1). The CD spectrum with only 100 mM NaCl has positive and negative peaks near 295 and 265 nm, respectively, indicating that the structure of d($G_4T_4G_4$) under this condition is the antiparallel G-quadruplex. In contrast, the spectrum in the presence of 100 mM NaCl and 50 mM $CaCl_2$ has positive and negative peaks near 260 and 240 nm, respectively, typical of the parallel G-quadruplex (*20*). The CD spectrum in the presence of a polycation (putrescine, cadaverine, or spermine) has negative and positive peaks near 295 and 265 nm, respectively, indicating that the structures of d($G_4T_4G_4$) under

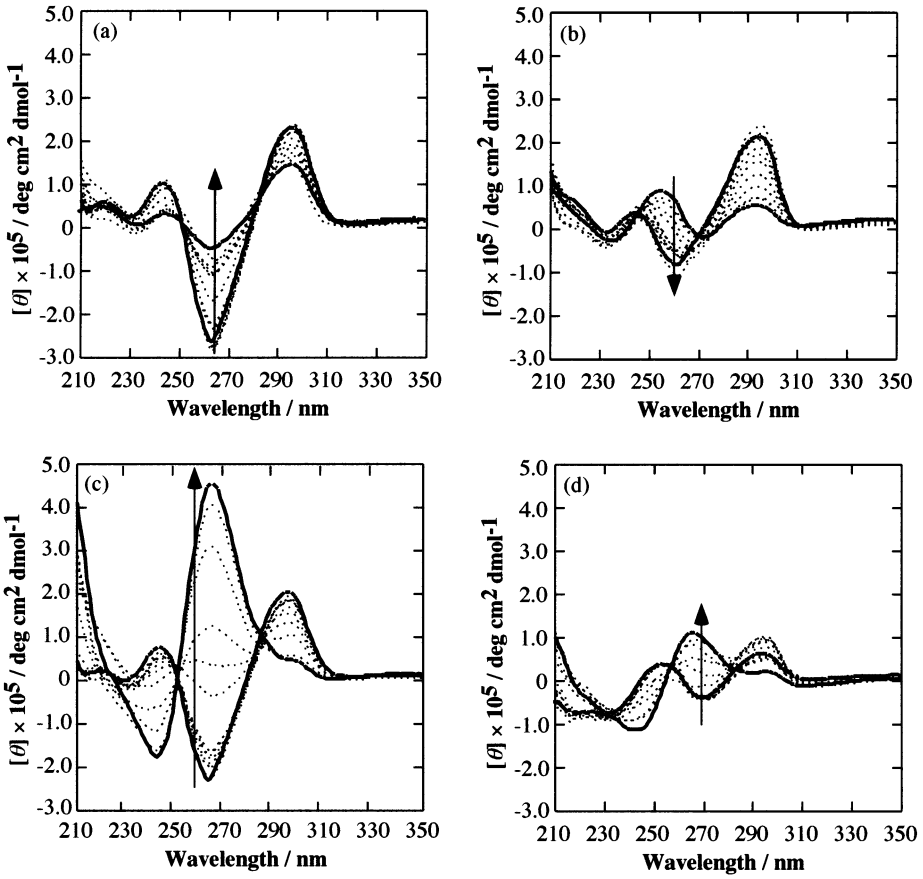

FIGURE 3: CD spectra of 50 μM d($G_4T_4G_4$) at 5 °C in buffers containing 50 mM MES and various concentrations of (a) putrescine with 100 mM NaCl, (b) putrescine without NaCl, (c) PEG with 100 mM NaCl, and (d) PEG without 100 mM NaCl (from the lower spectrum to the upper one at 260 nm: 0 M, 10 μM, 100 μM, 1 mM, 10 mM, 100 mM, 500 mM, 700 mM, 1.0 M, 1.2 M, 1.5 M, 1.7 M, and 2.0 M). CD spectra with 0 and 2 M putrescine or PEG are shown using thick lines for clarity.

these conditions are antiparallel G-quadruplexes, while the CD spectrum in the presence of 2 M neutral polymer (PEG or glycerol) has a positive peak near 260 nm and a negative peak near 240 nm, indicating that the structures under these conditions are parallel G-quadruplexes. These results indicate that molecular crowding with neutral polymers induces a structural transition from the antiparallel to the parallel G-quadruplex of d($G_4T_4G_4$). It has previously been reported that a crowding condition with PEG induced the folding of DNAs (*30*−*33*). We also found that the structure of d($G_4T_4G_4T_4G_4T_4G_4$) in the presence of 2 M PEG and 100 mM NaCl is a parallel G-quadruplex (Figure 2b), although it was reported that it forms an intramolecular antiparallel G-quadruplex in the presence of only NaCl (*34*). These results lead to the conclusion that molecular crowding with a high concentration of PEG generally induces the structural transition from an antiparallel to a parallel G-quadruplex, although molecular crowding with polycations does not alter the antiparallel G-quadruplex structure.

Effect of Polycation and PEG Concentrations on the Structural Transition of d($G_4T_4G_4$). The effect of molecular crowding induced by polycations and neutral polymers on the G-quadruplex structure of d($G_4T_4G_4$) was investigated by CD titration experiments using putrescine and PEG as shown in Figure 3. Panels a and b of Figure 3 show the CD spectra of 50 μM d($G_4T_4G_4$) with various concentrations of putrescine (from 0 to 2 M) in buffers containing 50 mM MES (pH 6.1) in the presence and absence of 100 mM NaCl, respectively. Figure 3a shows that the CD spectrum with 2 M putrescine in NaCl still has positive and negative peaks near 295 and 265 nm. This indicates that molecular crowding with putrescine does not alter the antiparallel G-quadruplex structure although it decreases its intensity. Figure 3b shows that a high concentration of putrescine induces positive and negative CD intensity near 295 and 265 nm that is similar to the spectrum in the presence of 2 M putrescine and 100 mM NaCl (Figure 3a). Because the equilibrium constants for macromolecular associations are generally increased by 2−3 orders of magnitude (*35*), the high concentration of putrescine can induce the unstable antiparallel G-quadruplex by Li^+, which is used for adjustment of the pH value, even in the absence of Na^+ (T_m = 41.6 °C). However, d($G_4T_4G_4$) cannot form the antiparallel G-quadruplex under a non-crowding condition without Na^+. This confirms that the structure induced by the high concentration of putrescine is still the antiparallel G-quadruplex. Panels c and d of Figure 3, however, show the CD spectra of 50 μM d($G_4T_4G_4$) in buffers containing 50 mM MES (pH 6.1) and various concentrations of PEG (from 0 mM to 2 M) in the presence and absence of 100 mM NaCl, respectively. The CD intensity change at 260 nm induced by 2 M PEG with 100 mM NaCl (5.2×10^5 deg cm^2 dmol^{-1}) is eight times that without NaCl (0.64×10^5 deg cm^2 dmol^{-1}). This is due to the structural formation of DNA requiring certain cations such as Na^+, although Li^+ and the high concentration of PEG may induce the parallel G-quadruplex (T_m = 72.1 °C), as in the case of the antiparallel G-quadruplex shown in Figure 3b. The CD spectra in the PEG titration have isodichroic points, indicating

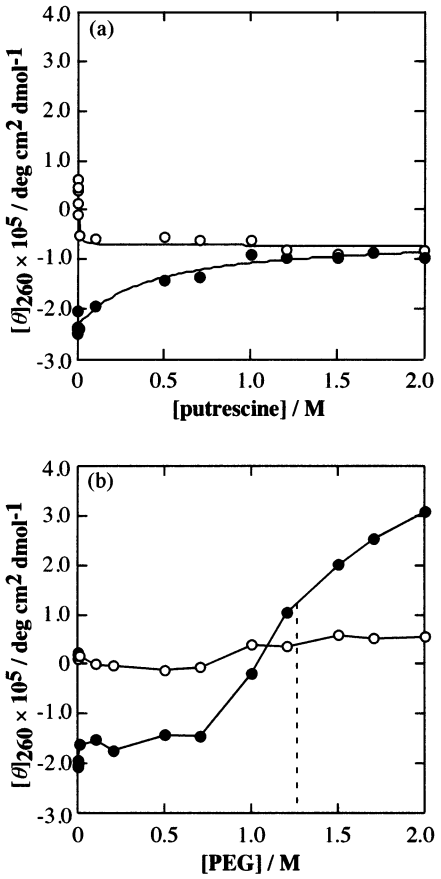

FIGURE 4: CD intensity at 260 nm of 50 μM d($G_4T_4G_4$) at 5 °C for various concentrations of (a) putrescine and (b) PEG in buffers containing 50 mM MES in the presence (shaded circle) and absence (unshaded circle) of 100 mM NaCl.

that the structural transition induced by molecular crowding with various concentrations of PEG is between the two states.

The CD intensity at 260 nm is useful for detecting the structural transition between antiparallel and parallel G-quadruplexes because the typical CD spectra of the parallel and antiparallel G-quadruplexes show large positive and negative peaks near 260 nm, respectively (*20*). The CD intensity change at 260 nm of 50 μM d($G_4T_4G_4$) during the putrescine titrations in the absence and presence of 100 mM NaCl is shown in Figure 4a. The binding constants, K_a, of putrescine for d($G_4T_4G_4$) in the absence and presence of 100 mM NaCl were calculated with the equation:

$$CD = CD_{F-I}\{K_a[S]/(1 + K_a[S])\} + CD_I \qquad (4)$$

Here CD, CD_{F-I}, and CD_I are the observed CD intensity, the magnitude of the CD intensity change, and the initial CD intensity, respectively, at 260 nm. [S] is the concentration of solute (putrescine). The nonlinear least-squares fit of the data to eq 4 is also shown in Figure 4a. The calculated K_a values in the presence and absence of NaCl are listed in Table 1. From the value of K_a, the free energy change at 5 °C can be estimated with the equation:

$$\Delta G^\circ_5 = -RT \ln K_a \qquad (5)$$

Here R and T are the gas constant and temperature (278.15 K). The ΔG°_5 values showed that the putrescine−d($G_4T_4G_4$) interaction is thermodynamically favorable (Table 1), in

Table 1: Binding Constant and Free Energy Change for the Interaction of Putrescine and PEG with d($G_4T_4G_4$)[a]

	in the absence of 100 mM NaCl		in the presence of 100 mM NaCl	
	K_a (M^{-1})	ΔG°_5 (kcal mol^{-1})[b]	K_a (M^{-1})	ΔG°_5 (kcal mol^{-1})[b]
putrescine	277 ± 148	−3.11 ± 0.23	2.50 ± 1.00	−0.51 ± 0.18
PEG	nd	nd	0.77	0.15

[a] The binding constant (K_a) was estimated at 5 °C. [b] The free energy change (ΔG°_5) was calculated from K_a at 5 °C with the equation $\Delta G^\circ_5 = -RT \ln K_a$, where R and T are the gas constant and temperature (278.15 K), respectively.

contrast to the results of the PEG titration experiments as described below. Because putrescine and DNA oligonucleotide have positive and negative charges, respectively, putrescine can bind to DNA via electrostatic interactions. The K_a value in the absence of NaCl is 100 times that in the presence of 100 mM NaCl (Table 1). This is due to excess Na^+ inhibiting the electrostatic interactions between putrescine and the phosphate groups of DNA. This coordination of the polycation to DNA phosphate groups may inhibit the structural transition from the antiparallel to the parallel G-quadruplex of d($G_4T_4G_4$) that appears in molecular crowding with PEG as shown in Figures 3c and 4b.

Figure 4b shows the CD intensities of 50 μM d($G_4T_4G_4$) at 260 nm during the PEG titration in the absence and presence of 100 mM NaCl. The results suggest that the structural transition from antiparallel to parallel G-quadruplexes of d($G_4T_4G_4$) occurred continuously and cooperatively with the PEG titration in the presence of 100 mM NaCl, although the titration curve in the absence of NaCl did not show a drastic change. At PEG concentrations lower than 0.7 M in the presence of 100 mM NaCl, a significant effect on the structure of d($G_4T_4G_4$) was not observed. The midpoint of the structural transition induced by molecular crowding with PEG in the presence of 100 mM NaCl is about 1.3 M as shown in Figure 4b, and therefore, the observed binding constant, K_a, of PEG for d($G_4T_4G_4$) is estimated to be 0.77 M^{-1}. From this value, ΔG°_5 is calculated to be +0.15 kcal mol^{-1} from eq 5 (Table 1). These results indicate that the interaction between d($G_4T_4G_4$) and PEG is thermodynamically unfavorable, and therefore, the structural transition from the antiparallel to parallel G-quadruplex was induced by the excluded volume, a major factor in molecular crowding (*1*, *2*).

Thermodynamic Stability of the G-Quadruplexes under the Crowding Condition. The effect of the molecular crowding induced by putrescine and PEG on the stability of G-quadruplex structures was also investigated. A typical CD spectrum of an antiparallel G-quadruplex has a large positive peak near 295 nm (*25*), and this is useful for detecting an antiparallel G-quadruplex formation. Figure 5a shows the normalized CD intensity of 50 μM d($G_4T_4G_4$) at 295 nm against temperature, without or with 2 M putrescine, in a buffer containing 100 mM NaCl and 50 mM MES (pH 6.1). The thermodynamic parameters calculated from these melting curves using the curve fitting procedure are listed in Table 2. These parameters show that molecular crowding with 2 M putrescine destabilizes the antiparallel G-quadruplex ($-\Delta G^\circ_{25}$ decreased from 28 to 22 kcal mol^{-1}). Moreover, on the basis of the enthalpy change (ΔH°) and entropy

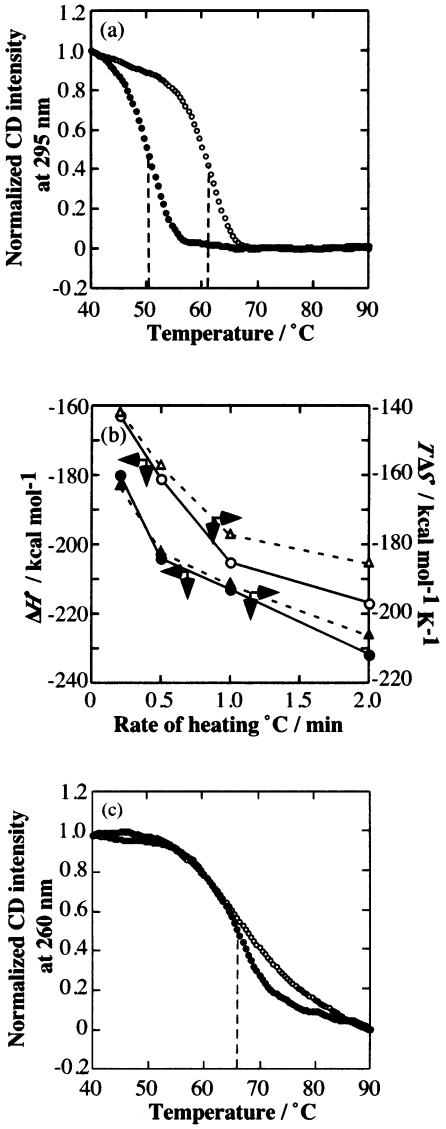

FIGURE 5: (a) Normalized CD intensity changes in 50 μM d($G_4T_4G_4$) at 295 nm against temperature in a buffer containing 100 mM NaCl and 50 mM MES in the absence (unshaded circle) or presence (shaded circle) of 2 M putrescine. (b) Thermodynamic parameters of 50 μM d($G_4T_4G_4$) calculated from the melting curves with the various rates of sample heating. Unshaded and shaded circles indicate $\Delta H°$ values in the absence and presence of 2 M putrescine, respectively. Unshaded and shaded triangles indicate $T\Delta S°$ values in the absence and presence of 2 M putrescine, respectively. (c) Normalized CD intensity changes in 50 μM d($G_4T_4G_4$) at 260 nm against temperature in a buffer containing 100 mM NaCl, 50 mM MES, and 50 mM $CaCl_2$ (opened circle) or 2 M PEG (closed circle).

Table 2: Thermodynamic Parameters for G-Quadruplex Formation of d($G_4T_4G_4$) at 25 °C[a]

condition	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1})	$\Delta G°_{25}$ (kcal mol^{-1})	T_m[b] (°C)
100 mM NaCl	-205 ± 8	-177 ± 7	-28 ± 2	61.4
2 M putrescine + 100 mM NaCl	-213 ± 11	-191 ± 10	-22 ± 2	50.3

[a] All experiments were carried out in a buffer containing 50 mM MES (pH 6.1). [b] Melting temperatures were calculated at a total strand concentration of 50 μM d($G_4T_4G_4$).

change ($T\Delta S°$) in the antiparallel G-quadruplex formation of d($G_4T_4G_4$) under crowding and noncrowding conditions with 2 M putrescine, the $\Delta\Delta H°$ [$=\Delta H°$(with putrescine) − $\Delta H°$(without putrescine)] and $-T\Delta\Delta S°$ [$=-T\Delta S°$(with putrescine) + $T\Delta S°$(without putrescine)] were −8 and 14 kcal mol^{-1}. Therefore, the destabilization effect of molecular crowding on the antiparallel G-quadruplex structure formation is predominantly induced by the entropy decrement. It was reported that the thermal denaturation profile of the G-quadruplex is sensitive to the rate of heating (*26*). Therefore, we also investigated the effect of the rate of heating on the thermodynamic parameters of d($G_4T_4G_4$), without or with 2 M putrescine, in a buffer containing 100 mM NaCl and 50 mM MES (pH 6.1). The results showed that both $\Delta H°$ and $\Delta S°$ with and without putrescine decrease with the increasing rate of heating from 0.2 to 2.0 °C/min, and then the $\Delta\Delta H°$ and $-T\Delta\Delta S°$ values discussed here were not changed with the heating rate (Figure 5b). Because d($G_4T_4G_4$) undergoes a two-state transition between a single strand and the antiparallel G-quadruplex (*27*), the entropy decrement in the antiparallel G-quadruplex formation results from the larger conformational flexibility of single strands compared to the larger conformational rigidity of the G-quadruplex. Macromolecules are distributed less randomly by molecular crowding, and the configurational entropy of the macromolecular solute species becomes smaller (*35*). Thus, in this study, molecular crowding can promote the structural rigidity of the G-quadruplex by the effect of excluded volume. The other consideration for the entropy decrement is that the molecular crowding should affect the activity of small molecules such as cations and water, which may have an effect on the entropy value of the system. However, it was reported that the activity coefficient increases significantly only for molecules larger than a molecular weight of 1000 (*36*, *37*). Thus, molecular crowding does not have a large effect on the activity of the small molecules. These previous results and considerations lead to the conclusion that the entropy decrement in the antiparallel G-quadruplex formation (Table 2) is due to the larger conformational rigidity of the G-quadruplex. The enthalpy change in the presence of 2 M putrescine, however, is smaller than that in the absence of putrescine as described above, indicating that the putrescine−d($G_4T_4G_4$) interaction is stable even with molecular crowding. It should be noted that, in an interaction between solutes such as putrescine−d($G_4T_4G_4$), electrostatic interaction is a major factor in molecular crowding as well as the excluded volume effect (*37*, *38*). These energetic considerations are comparable with the results of the putrescine titration experiments in the presence and absence of NaCl as shown in Figure 4a. The thermodynamic parameters listed in Table 2 thus revealed, quantitatively and individually, the effect of excluded volume and chemical interaction. Recently, Pielak and co-workers investigated the effect of molecular crowding by saccharides on protein binding by comparing theoretical results with their experimental results (*38*, *39*). They showed the thermodynamic parameters of protein−protein binding under crowding conditions with saccharides and indicated that both the excluded volume and chemical interaction between solutes should be important for analysis of the molecular crowding. These results, together with our present results, lead to the conclusion that the thermodynamic parameters are essential for investigating the effect of molecular crowding on the binding and stability of biomacromolecules such as proteins and nucleic acids.

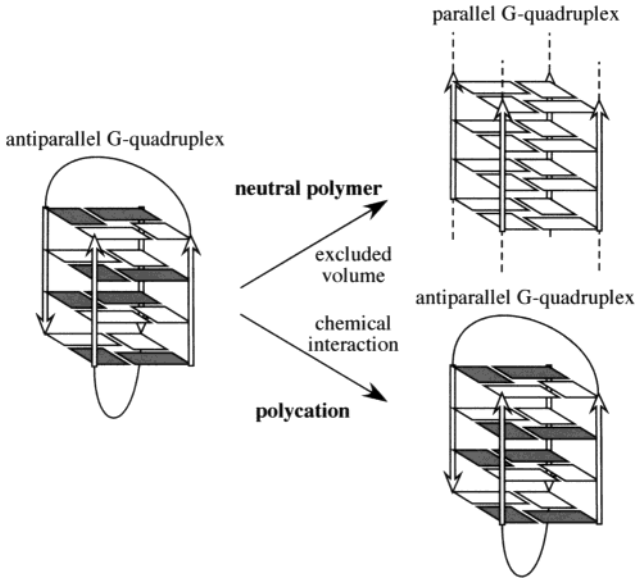

FIGURE 6: Schematic illustrations of the effects of macromolecular crowding with neutral polymers such as PEG and with the polycations such as putrescine in the presence of 100 mM NaCl. The neutral polymers induce the parallel G-quadruplex structure due to excluded volume, while the polycations do not alter the antiparallel G-quadruplex structure due to electrostatic interaction.

Figure 5c shows the normalized CD intensity of 50 μM $d(G_4T_4G_4)$ at 260 nm against temperature in the buffers containing 100 mM NaCl and 50 mM MES (pH 6.1) in the presence of 50 mM $CaCl_2$ or 2 M PEG. We previously showed that the parallel-stranded G-quadruplex induced by 50 mM $CaCl_2$ includes not only a homogeneous four-stranded parallel G-quadruplex but also a highly ordered association such as a G-wire (*20*). Therefore, although the thermal denaturation of $d(G_4T_4G_4)$ under these conditions exhibits an apparent two-state transition, it is difficult to determine thermodynamic parameters such as those shown in Table 2. However, the melting temperature of the parallel G-quadruplex in the presence of 50 mM $CaCl_2$ was estimated to be 66.0 °C and that with 2 M PEG, 65.5 °C. This indicates that the stability of the parallel G-quadruplex induced by molecular crowding with PEG is almost the same as that induced by Ca^{2+}.

Driving Force of the Structural Transition Induced by the Molecular Crowding with PEG. Next, we discuss the effect of the excluded volume and chemical interaction on the structural transition from the antiparallel to the parallel G-quadruplex. The chemical interaction between PEG and $d(G_4T_4G_4)$ is thermodynamically unfavorable (Table 1); therefore, the structural transition induced by PEG is dependent on the effect of the excluded volume derived from the high concentration of PEG. The antiparallel G-quadruplex structure of $d(G_4T_4G_4)$, in the presence of Na^+, has a syn−anti−syn−anti conformation in all four guanine repeats, and the conformation in the G-quartet plane is G(syn)·G(syn)·G(anti)·G(anti) (Figure 6); thus, the antiparallel G-quadruplex has one narrow, two medium, and one wide grooves (*23*). On the other hand, all of the guanines of a parallel four-stranded G-quadruplex are in the anti glycoside conformation (Figure 6), and therefore, all four grooves are identical (*40*). Although the structure of the loop region with T repeats of the parallel-stranded G-quadruplex of $d(G_4T_4G_4)$ has not been determined, the volume of the G-quartet region of the parallel-stranded G-quadruplex may be smaller than that of the antiparallel G-quadruplex because of its all-anti conformation. On the other hand, it was previously reported that the excluded volume increasingly favors monomer association to form dimers, trimers, tetramers, and then polymers, because crowded solutions favor the association of reactants and reduction in the reactant volume (*41*, *42*). Therefore, the effect of excluded volume with a high concentration of PEG on the G-quadruplex structure should lead to the structural transition from the antiparallel to the parallel G-quadruplex as shown in Figure 6. In contrast, molecular crowding with 2 M putrescine does not induce the structural transition (Figure 6), which is partly due to the coordination of putrescine to the phosphate group of $d(G_4T_4G_4)$, as discussed above.

Biological Significance of the Structural Transition Induced by the Molecular Crowding. Because double-stranded DNA is generally formed with Watson−Crick base pairs in cells, the formation of a G-quadruplex with Hoogsteen base pairs may lead to unusual local unwinding and separation of strands. However, the 3′ single-stranded overhang at the end of chromosomes (telomere overhang) can form one- or two-stranded antiparallel G-quadruplexes without strand separation of the DNA duplex. It has been proposed that the structural switching between antiparallel and parallel G-quadruplexes of the guanine-rich telomere DNAs plays a vital role in chromosome association (*21*, *22*). It has also been proposed that biological processes concerning cell life, such as apoptosis and meiosis of eucaryotic cells, are also connected with guanine-rich oligonucleotides such as the telomere (*43*) and therefore the G-quadruplex structure. While there have been many in vitro studies of G-quadruplex structures in simple buffer systems, these studies have not simulated the crowding conditions in vivo. It has previously been reported that significant increases in the level of molecular crowding could result from age-related effects: a reduction in cell volume and the inhibition of protein degradation (*44*). Cell volume change has also been observed in Schwann cells through the cell cycle (*45*). We have shown, in the present study, that molecular crowding critically affects the structure of the G-quadruplex.

CONCLUSION

Several studies were developed in an effort to quantify the effects of high solute concentrations on the free energy of biological equilibration. We have shown that molecular crowding with neutral polymers induces the structural transition from the antiparallel to the parallel G-quadruplex, although molecular crowding with the polycations does not. Thermodynamic parameters showed quantitatively that the volume excluded by solutes and the chemical interaction between DNA and the solutes affect the structure of G-quadruplex through entropy and enthalpy changes, respectively, and that the excluded volume and chemical interaction partially cancel each other in determining the G-quadruplex structure under molecular crowding with a polycation. In a living cell, there are various solution components including biomacromolecules such as proteins and nucleic acids, salts, substrates, and cofactors. Our results show that a combination of biomacromolecules and other solutes, which induce molecular crowding, should be con-

sidered in the investigation of biomacromolecular structure, stability, and function in a living cell.

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BI020412F