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Pressure-dependent formation of i-motif and G-quadruplex DNA structures

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Pressure is an important physical stimulus that can influence cell fate by causing structural changes in biomolecules such as DNA. We investigated the effect of high pressure on the folding of duplex, DNA i-motif, and G-quadruplex (G4) structures; the non-canonical structures may be modulators of expression of genes involved in cancer progression. The i-motif structure was stabilized by high pressure, and the G4 was destabilized. The melting temperature of intramolecular i-motif formed by 5'-dCGG$(CCT)_{10}$CGG-3' increased from 38.8 °C at atmospheric pressure to 61.5 °C at 400 MPa. This effect was also observed in the presence of 40 wt% ethylene glycol, a crowding agent. In the presence of 40 wt% ethylene glycol, the G4 structure was less destabilized than in the absence of the crowding agent. *P-T* stability diagrams of duplex DNA with a telomeric sequence indicated that the duplex is more stable than G4 and i-motif structures under low pressure, but the i-motif dominates the structural composition under higher pressure. In crowding conditions, the *P-T* diagrams indicated that duplex does not form under high pressure, and i-motif and G4 structures dominate. Our findings imply that temperature regulates formation of the duplex structure, whereas pressure triggers the formation of non-canonical DNA structures like i-motif and G4. These results suggest that pressure impacts the function of nucleic acids by stabilizing non-canonical structures; this may be relevant to deep sea organisms and during evolution in prebiotic conditions.

Introduction

Living cells respond to physical stimuli like temperature and pressure and to chemical stimuli (e.g., pH, oxidation, and small molecules) with changes in the patterns of gene expressions. For example, the fates of stem cells can be controlled by the external physical environments.[1, 2] In the process of the response to external stimuli, the structures of biomolecules like DNA may be altered. DNA tetraplexes are non-canonical structures that are responsive to physical and chemical stimuli. Guanine-rich sequences are able to form G-quadruplexes (G4); this structure is composed of G-quartets stabilized by Hoogsteen base pairings.[3, 4] The stability of G4s depends on metal ions present in solution, and molecular crowding agents like poly(ethylene glycol) stabilize these structures. Sequences capable of forming G4s are found in telomeres and in promoter regions of known oncogenes. Thus, G4 formation may regulate expression of genes involved in cancer progression and other diseases.[5]

Under certain conditions, tandem repeats of cytosine-rich sequences may also form a tetrad conformation called i-motif.[6-8] An intramolecular i-motif forms upon parallel interaction of four C-rich strands and has three loops. The i-motif is stabilized by hydrogen bonding between cytosine and protonated cytosine $(C:C^+)$[9-11], and therefore, acidic conditions stabilize the i-motif structure.[12] Molecular crowding conditions stabilize the i-motif structure by increasing the pK_a of cytosine.[13] Although the biological significance of i-motif is still unclear, this unique structure has attracted attention because C-rich sequences are found in promoter regions of about 40% of human genes.[14, 15] Recently, it was reported that an i-motif forming sequence located in the *BCL2* promoter region regulates transcription of this pro-survival oncoprotein, and the importance of the i-motif structure was confirmed by the addition of a molecule that binds specifically to this structure.[16] Another report showed that an i-motif-forming oligonucleotide stabilized by a single wall carbon nanotube inhibits telomerase activity.[17, 18]

Physical stimuli might also induce i-motif formation. High pressure induces changes in nucleic acids, including changes in hydration[19] and hydrogen bonding [20, 21], and induces certain structural transitions [22, 23]. Although the stability of canonical DNA duplexes changes very little when pressure is increased, some non-canonical DNA structures are significantly affected.[24-27] Chalikian's group and our group recently found that G4s are destabilized upon increase of pressure due to the

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larger partial molar volume of the G4 relative to the random coil.[26,27] High pressure also results in protonation. Aminoacylated tRNA is stabilized under high pressure even at neutral pH, which implies protonation of water molecules.[28] Thus, volumetric and chemical changes induced by high pressure may alter stability of the i-motif (Fig. 1).

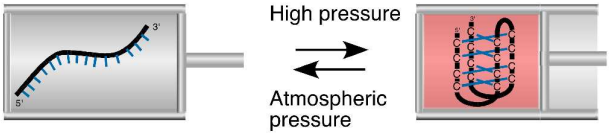

Fig. 1 Schematic illustration of conformational equilibrium monitored in this study. Pressure affects the stability of i-motif structure due to volumetric and protonation changes.

In this study, we investigated the effect of pressure on the formation of G4 and i-motif DNA structures. UV melting analysis demonstrated that thermal stability of the i-motif structure increased with increasing pressure in phosphate buffer at near neutral pH; pressure had the opposite effect on the G4 structure. Pressure facilitated the protonation of cytosine bases by lowering the pH in the solution. That this was the main mechanism for stabilization of i-motif DNA was supported by the result that the stability of the i-motif structure did not differ with and without the crowding agent ethylene glycol over the range of pressures tested. The *P-T* stability diagram of a DNA duplex with a telomeric DNA showed that the C-rich strand dominated the structural equilibrium under high pressure. Our results suggest that non-canonical structures are responsive to pressure and temperature and that increases in pressure that destabilize the duplex structure may allow non-canonical structures to regulate gene expression. The differences in responses of nucleic acids to pressure and temperature may have contributed to the evolution of the life and may exert control over gene expression in modern organisms through effects on DNA structure.

Results

The thermal stabilities of G-quadruplex and i-motif structures under high pressure.

To examine the effect of pressure on the thermal stability of G4 and i-motif structures, UV melting was performed under pressures ranging from atmospheric pressure (0.1 MPa) to 400 MPa in a solution containing 10 mM NaH_2PO_4 (pH 6.0, adjusted at 0.1 MPa) and 1 mM Na_2EDTA. As a model for G4 formation, we studied the oligonucleotide 5'-d(AG_3T_2)$_3AG_3$-3', referred to as HT DNA in this manuscript, a sequence from the human telomere. The intramolecular anti-parallel G4 structure formed by this DNA oligonucleotide in Na^+ buffer is well characterized.[28] As an i-motif model, we used a DNA oligonucleotide with ten repeats of CCT, 5'-dCGG(CCT)$_{10}$CGG-3', referred to as (CCT)$_{10}$ DNA here.[13] Repeated sequences similar to this are found in genes implicated in triplet repeat expansion diseases.[29] (CCT)$_{10}$ DNA forms an intramolecular i-motif structure at 0.1 MPa, as UV melting had no concentration dependence and the CD signal was typical of previously characterized i-motif structures with a positive band near 290 nm and a negative one around 265 nm (Supporting Information, Fig. S1 and S2).

Fig. 2A shows typical UV melting curves of G_4T_4 DNA assessed by monitoring absorbance at 295 nm as a function of temperature under 0.1, 50, and 100 MPa pressure. At 0.1 MPa, melting began at approximately 50 °C, and the curves shifted toward lower temperature with increasing pressure. We also observed pressure-induced destabilization of the G4 structure adopted by 5'-d(G_4T_4)$_3G_4$-3', referred to as G_4T_4 DNA in this manuscript, a sequence from the telomere of *Oxytricha nova* (Fig S3). Thus, this G4 structure was destabilized under high pressure, as observed for other G4 forming DNAs previously.[26, 27, 30, 31] Fig. 2B shows typical UV melting curves of (CCT)$_{10}$ DNA under 0.1, 100, 200, 300, and 400 MPa. At 0.1 MPa, the UV absorbance began to decrease at approximately 25 °C and was constant at temperatures higher than 50 °C. Interestingly, when pressure was increased, the melting curves shifted significantly toward higher temperatures. The melting curves of other i-motif forming DNAs with sequence complementary to HT DNA, 5'-d$C_3T(A_2C_3T)_3$-3' (cHT DNA), and sequence complementary to G_4T_4 DNA, 5'-d(C_4A_4)$_3C_4$-3' (C_4A_4 DNA), also shifted toward higher temperatures with increasing pressure (Fig. S4A and S4B, respectively). Thus, pressure enhanced the stability of the i-motif structure and decreased the stability of the G4 structure.

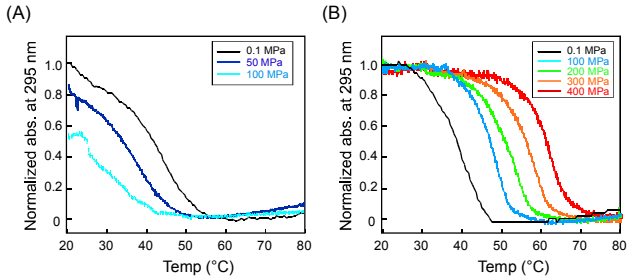

Fig. 2 Effect of pressure on the transition of DNA structure. (A) The changes of absorbance at 295 nm of 20 μM HT DNA under 0.1 MPa (black), 50 MPa (blue), and 100 MPa (light blue). (B) The changes of absorbance at 295 nm of (CCT)$_{10}$ DNA under 0.1 MPa (black), 100 MPa (light blue), 200 MPa (green), 300 MPa (orange), and 400 MPa (red). Each solution was buffered with 10 mM Na_2HPO_4 and 1 mM Na_2EDTA, pH 6.0 (adjusted at 0.1 MPa).

The pressure effect on the stability of (CCT)$_{10}$ DNA was also studied at pH 7.0 (adjusted at 0.1 MPa). At this pH at atmospheric pressure, the stability of the i-motif form of (CCT)$_{10}$ DNA was very low (Fig. S4C). At higher pressures, the UV melting curves shifted toward higher temperature. Thus, high pressure induced the formation of the i-motif structure even at pH 7.0. In the case of cHT DNA, only a small amount of i-motif formation could be detected under 400 MPa of

pressure (Fig S4D). This difference is probably due to the fact that the cHT oligonucleotide forms fewer C:C$^+$ base pairs than $(CCT)_{10}$. The melting temperatures (T_ms) at pH 6.0 were determined from the UV melting curves and are shown in Table 1. The T_m of $(CCT)_{10}$ DNA in pH 6.0 solution at 0.1 MPa was 38.8 °C, whereas the value of $(CCT)_{10}$ DNA in the same solution at 400 MPa was 61.5 °C (Table 1). The T_ms of $(CCT)_{10}$ DNA increased almost linearly with pressure (Fig. S5). The value of $\Delta T_m/\Delta P$ was 5.5 x 10^{-2} K MPa^{-1}; this is significantly higher than the previously reported values for $\Delta T_m/\Delta P$ for the duplexes of calf thymus DNA and poly[d(A-T)] at low salt concentrations (0.46 x 10^{-2} K MPa^{-1} in 20 mM KCl and 0.36 x 10^{-2} K MPa^{-1} in 20 mM NaCl, respectively. [30, 31]

Table 1. Melting temperatures (T_m) for $(CCT)_{10}$ DNA as a function of pressure[a].

Pressure (MPa)	T_m (°C)
0.1	38.8 ± 0.9
100	45.9 ± 1.1
200	51.7 ± 0.3
300	56.0 ± 0.8
400	61.5 ± 0.7

[a] UV melting of 20 μM $(CCT)_{10}$ DNA was measured in 10 mM Na_2HPO_4, 1 mM EDTA pH 6.0. The pH of the solution was adjusted to 6.0 at 0.1 MPa.

Protonation of i-motif forming sequences as a function of pressure

Since the i-motif structure depends on C:C$^+$ base pairing, high pressure may facilitate the protonation of cytosine. To investigate whether the protonation of cytosine was triggered by pressure, we analyzed the spectrum of methyl red (pK_a = 5.1), which shifts with changing pH due to protonation. When pH was decreased from 7 to 5, the methyl red absorbance at 440 nm decreased, whereas the absorbance increased at 550 nm (Fig. S6A). Similar changes in the spectra were observed as pressure was increased (Fig. S6B). An isosbestic point was not detected; this may be due to a decrease in extinction coefficient due to the pressure increase. The ratio of A_{440}/A_{550} at pH 6.0 under 400 MPa was 1.67, similar to the value at pH 5.0 under the 0.1 MPa of 1.6 (Table S1). The ratios at 300 MPa (1.65), 200 MPa (2.64), and 100 MPa (4.62) were similar to those at pH 5.1 (1.74), pH 5.3 (2.30), and pH 5.7 (4.62), respectively. The T_m of $(CCT)_{10}$ DNA at pH 5.1, 5.3, and 5.7 at 0.1 MPa were 56.8, 53.7, and 45.6 °C (Table S2). These values correlate with T_m values at 300, 200, and 100 MPa, respectively (Table 1). At the 0.1 MPa, the values of $\log(A_{440}/A_{550})$ and T_m values of $(CCT)_{10}$ DNA as a function of pH were linearly correlated (Figs. S6C and S6D). By monitoring of the absorbance due to methyl red, we showed that the pH of MES buffer did not change as pressure was increased (Fig S7A and S7B). We therefore analysed i-motif stability in MES (Fig. S7C). Melting temperatures were 47.6 at 0.1 MPa and 47.0 at 200 MPa. Therefore, the pressure does not impact the stability of i-motif DNA, whereas pressure-induced pH changes do impact i-motif stability.

Pressure induction for the structural switching of i-motif DNA

Next, we examined whether a pressure change could induce formation of the i-motif structure. At 25 °C, absorbance increased linearly with pressure (Fig. 3). The increase in absorbance over this pressure range was similar when a single-stranded DNA was evaluated (data not shown). As pressure was released at a constant 25 °C, the absorbance decreased linearly to return, within experimental error, to the initial value. Thus, we concluded that absorbance changes as a function of pressure results from the effective increase in oligonucleotide concentration due to the compression of solution and/or changes in extinction coefficient that depend on pressure. We then performed the same experiment at 50 °C. The absorbance changed more significantly than it did at 25 °C. The curve as pressure was released was clearly sigmoidal (Fig. 3). The value of $P_{1/2}$ where folded and unfolded DNAs are present at a 1 to 1 ratio was 230 MPa. Thus, i-motif formation can be induced by a pressure change. There is one previous report of the unfolding of a DNA duplex induced by a pressure increase.[32]

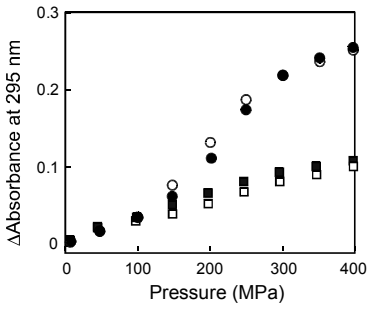

Fig. 3 Pressure dependent changes of UV absorbance of $(CCT)_{10}$ DNA at 25 °C (square) and 50 °C (circle) at pH 6.0 (at 0.1 MPa). Closed and opened symbols indicated the values measured during pressure increase and pressure decrease, respectively.

Pressure effect on the formation of i-motif DNA in the molecular crowding conditions

The binding of crowding agents, such as ethylene glycol (EG), to biomolecules induces changes in hydration state and cavity formation.[33] Molecular crowding agents destabilize Watson-Crick base pairs but stabilize Hoogsteen base pairs due to changes in water activity and DNA hydration[34-37] and were previously shown to repress the destabilizing effect of high pressure on a G4 structure due to decreases in hydration volume.[27] Here we examined the UV melting of $(CCT)_{10}$ DNA in the presence of 40 wt% EG under several pressures at pH 6.0 (adjusted at 0.1 MPa). As shown in Fig. 4, the melting curves indicate that the stability of $(CCT)_{10}$ DNA increased with

increasing pressure in the presence of EG. The melting temperatures at each pressure are summarized in Table S3. The value of $\Delta T_m/\Delta P$ was 6.1 x 10^{-2} K MPa^{-1} (Fig. S8), which was similar to that in the absence of EG. These results indicate that changes in hydration volume of $(CCT)_{10}$ DNA do not contribute to the stability increase induced by high pressure. As our experiments in the pressure of EG mimic intracellular conditions, we conclude that pressure should have more impact on formation of the i-motif structure than on the G4 structure inside cells.

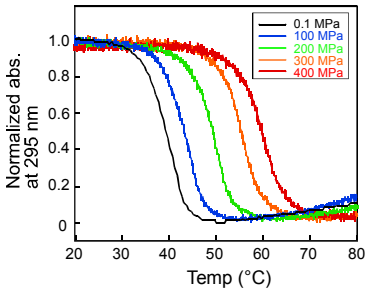

Fig. 4 UV melting curves of $(CCT)_{10}$ DNA in the presence of molecular crowding agent EG under 0.1, 100, 200, 300, and 400 MPa in a solution of 10 mM Na_2HPO_4, 1 mM Na_2EDTA at pH 6.0 (adjusted at 0.1 MPa) and 40 wt% EG.

P-T stability diagrams of telomeric DNA

In chromosomal DNA, sequences with i-motif-forming potential are paired with a complementary sequence capable of G4 formation. Thus, i-motif formation depends on stabilities of the duplex and the G4. We evaluated the structural transition from duplex to i-motif and G4 induced by high pressure. To detect duplex formation, we modified the C_4A_4 DNA oligonucleotide with a fluorophore at the 5' terminus and the G_4T_4 DNA with a quencher at the 3' terminus as described previously.[27, 38] The oligonucleotides were annealed at pH 5.5 by cooling from 80 °C to 37 °C under 10 MPa (minimum pressure for the high-pressure apparatus), 100, 200, 300, and 400 MPa in a solution of 10 mM Na_2HPO_4 and 1 mM Na_2EDTA. We then determined the ratio of formation of duplex from the normalized fluorescence intensity as duplex formation results in quenching of fluorescence (Fig. 5A). The percentage of the population that adopted of i-motif and G4 populations formed under each temperature and pressure were estimated based on the percent that did not form duplex and that showed structural transitions in normalized UV annealing curves (Fig. S9). Duplex formation was observed at low pressure and temperature (Fig. 5B). The highest population of G4 was observed at 0.1 MPa at 50 °C, but decreased at pressures higher than 200 MPa (Fig. 5C). G4 has a broader melting curve than duplex (Fig. S9), and the G4 DNA is destabilized at higher pressure.[26, 27] The i-motif became the major structure with increasing pressure at more than 200 MPa (Fig. 5D). At 37 °C, 88% of DNA formed duplex under 10 MPa, while only 9.7% of strands were involved in duplex at 400 MPa (Fig. 5E). At 37 °C under 10 MPa pressure, 3.7% and 5.8% of strands were estimated to form i-motif and G4, respectively. The fraction of strands involved in these structures increased with increasing pressure to 200 MPa (24% of strands in each non-canonical structure). At 400 MPa the population of i-motif increased up to 44%, but almost no G4 was estimated to be present.

To exclude the possibility that the destabilization of duplex enhanced the formation of i-motif, we carried out experiments in 30 mM Tris-HCl, pH 7.5, and 100 mM LiCl. Below 37 °C in these conditions, only duplex is formed because the presence of Li ion destabilized G4 and the slightly basic pH inhibited the formation of the i-motif structure. By analysis of fluorescence we found that the ratios of duplex formation by C_4A_4 and G_4T_4 systems were extremely high at ambient pressure (Fig. S10). Below 200 MPa very little duplex dissociation was observed. These results indicate that the pressure increase did not destabilize the duplex to promote i-motif structure, but rather the i-motif was stabilized and was able to inhibit duplex formation.

In the presence of 40 wt% EG, less duplex formed over the pressure range than in the absence of EG (Fig. 5F). The i-motif coexisted with G4 under all the pressures evaluated in the presence of EG (Figs. 5G and 5H). This is because the crowding agent stabilized the G4 structure[27], and i-motif stability was enhanced with increasing pressure. Only 19% of strands formed duplex at 37 °C under 10 MPa, and less than 10% were duplex at higher pressure (Fig. 5I).

Next we investigated the *P-T* diagrams of G_4T_4 in a solution of 10 mM K_2HPO_4 and 1 mM K_2EDTA; at ambient pressure, the G4 structure shows polymorphic dependent on the cosolute. Although G-quadruplex polymorphism under pressure has not been characterized, the stability of the G-quadruplex formed in K^+ was decreased with increasing pressure (Fig. S11) as was observed in Na^+. With increasing pressure in K^+, the G4 structure was destabilized (Fig. S12), as was observed in Na buffer condition. Therefore, the pressure affects all G4 structures adopted by G_4T_4 DNA similarly. In the *P-T* diagram, the population of duplex at 37 °C under 10 MPa was very small (1.8%). However, that under 100 MPa was increased to be 41.1%. Further increase of pressure decreased the population. On the other hand, at 37 °C under 0.1 MPa, G4 and i-motif were adopted by 38.6% and 33.5% of strands. At 37°C under 100 MPa, both populations were decreased, and 20.0 % and 28.6 % of strands would form G4 and i-motif. At 37°C under 200 MPa, little G4 was formed (7.0%), but 43.3% of strands adopted the i-motif structure. These results indicate that G4 and i-motif were less stable than duplex at 100 MPa. The equilibrium was driven by G4 formation under less than 100 MPa and by i-motif formation at higher pressures. In the presence of 40% EG, the formation of duplex was not observed under any conditions. This is because G4 was stabilized under crowded conditions (Fig. 5).

We also examined the *P-T* diagrams of cHT and HT DNA (Figs. S13 and S14). In the absence of EG with 10 mM Na_2HPO_4 and 1 mM Na_2EDTA, primarily duplex was formed at 37 °C and 10 MPa, but the percentage of duplex was significantly decreased at pressures up to 100 MPa. At above 100 MPa, the i-motif was the primary form. G4 formation was not be detected at more than 200 MPa. Thus, in contrast to the equilibrium populations of C_4A_4 and G_4T_4 structures, which were driven by the stability of G4, the equilibrium populations of human telomeric DNA were driven by the stability of the i-motif at pressures higher

than 100 MPa. In the presence of EG, little duplex was detected for any of the systems above 100 MPa.

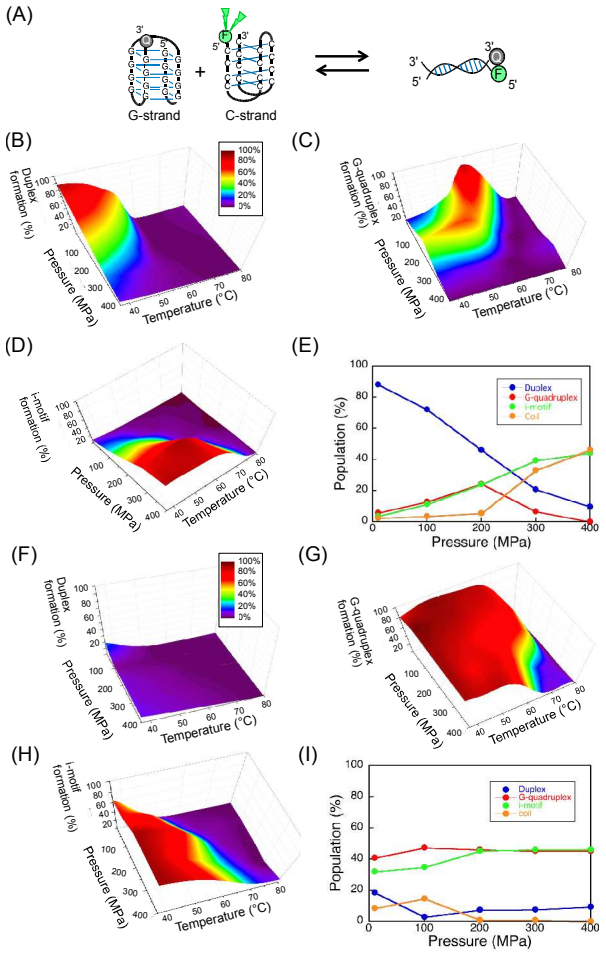

Fig. 5 Transitions of DNA structures driven by pressure and temperature. (A) Schematic illustration of florescence detection of duplex formation. (B-D) *P-T* stability diagrams of the normalized ratio of (B) duplex formation, (C) G-quadruplex formation by the G_4T_4 strand, and (D) i-motif formation by C_4A_4 strand in 10 mM Na_2HPO_4 and 1 mM Na_2EDTA at pH 5.5. (E) Percent of the population in duplex (blue), G-quadruplex (red), i-motif (green), and coil (orange) as a function of pressure at 37 °C in 10 mM Na_2HPO_4 and 1 mM Na_2EDTA at pH 5.5. (F-H) *P-T* stability diagrams of the normalized ratio of (F) duplex formation, (G) G-quadruplex formation, and (H) i-motif formation in 10 mM Na_2HPO_4 and 1 mM Na_2EDTA at pH 5.5 in 40 wt% EG. (I) Populations of duplex (blue), G-quadruplex (red), i-motif (green), and coil (orange) as a function of pressure at 37 °C in the presence of 40 wt% EG. In *P-T* diagrams, the color gradient indicates the percent of the population that adopts the structure.

Discussion

Pressure has little effect on the stability of duplex DNA.[30, 31] Pressure increases decrease G4 stability, because the volume change for the formation of this DNA structure (ΔV) is positive and large.[26, 27] In this study, we demonstrated an opposite effect of pressure on i-motif formation. Interestingly, the driving force of stabilization of the i-motif structure as pressure increases is due to more efficient protonation of C base as pH decreases with increasing pressure. Pressure presumably has a volumetric effect on phosphate buffer that enhances the acid dissociation constant of phosphate buffer. The characteristics of volumetric parameters for the i-motif structure should be different from those of G4 DNA. ΔV can be represented as the following equation 1.[39, 40]

$$\Delta V = \Delta V_M + \Delta V_T + \Delta V_I \quad (1)$$

The intrinsic volume V_M is the geometric volume of the solute molecules and is equivalent to the van der Waals volume of each atom of a molecule and cavity volumes. For conformational changes, ΔV_M is the change of cavity volume because van der Waals volume does not change unless covalent bonds are broken or formed. The other two factors are dependent on solvent: The thermal volume V_T is the volume of the layer of void space surrounding the solvent accessible surface of the solute molecules. This volume arises from the thermal motion of solute and solvent molecules. The interaction volume V_I is the solvent volume arising from the interaction between the solute and solvent; in water, the interaction leads to the formation of hydration shell composed of molecules with a higher density than bulk water. In the case of the formation of a double-stranded DNA (such as a duplex or a hairpin structure), the value of ΔV_M is almost zero because there are few cavities.[24] ΔV_T is negative due to the decrease of surface area, and ΔV_I is mostly negative due to hydration as pressure increases. Because the magnitudes of ΔV_T and ΔV_I are small, ΔV for double-stranded DNA is usually negative and small. Formation of G4 DNA has a positive ΔV_M due to large cavity volume of cavity in the quadruplex, a negative ΔV_T due to the decrease in surface area, and a positive ΔV_I due to dehydration. As a result, ΔV for G4 formation is large and positive. For i-motif formation the $\Delta T_m/\Delta P$ value of $(CCT)_{10}$ DNA was not altered by the presence of EG. Furthermore, from the analysis the melting curves, T_m and ΔH (-65.2 kcal mol^{-1} for 0% EG and -63.5 kcal mol^{-1} for 40% EG analyzed from each melting curve) in the absence and presence of EG were almost the same. ΔV value can be determined using the Clapeyron equation:

$$\Delta T_m/\Delta P = T_m \Delta V/\Delta H \quad (2)$$

For i-motif formation, values of $\Delta T_m/\Delta P$, T_m, and ΔH were almost the same in the presence and absence of EG, therefore ΔV will also be the same in the presence and absence of EG. This result suggests that the contribution of ΔV_I is very small for the formation of i-motif structure. Although there is no information about the volume of cavity of the i-motif DNA structure, we predict that ΔV_M value is relatively large and positive, cancelling the negative ΔV_T value, which results in the small magnitude of ΔV value of the i-motif formation. This prediction was supported by our analysis of i-motif formation in MES buffer. As shown in Fig. S7, the UV melting behaviour of $(CCT)_{10}$ in MES showed almost no dependence on pressure in the range from 0.1 to 200 MPa. As there was almost no change in pH in this pressure range, ΔV of i-motif formation of $(CCT)_{10}$ DNA is quite small.

Our data allow us to categorize nucleic acid structures from the viewpoint of pressure sensitivity. The G4 structures are destabilized under high pressure due to positive and large ΔV, but the destabilization is moderated in a crowded molecular environment. The i-motif structures were stabilized with increasing pressure without a volumetric contribution and crowding agents had little impact. Duplex DNA structures are slightly stabilized under high pressure and may possibly become more stable in the crowding condition due to hydration (opposite to the G4 type in ΔV_1).

As shown by the *P-T* diagrams (Fig. 5), i-motif and G4 structures dominant the structural landscape when both C-rich and G-rich strands are present under conditions of higher than physiological temperature and pressure. In a genomic DNA, the complement of a sequence with G4-forming potentially has the capability to adopt the i-motif structure; in these regions, G4 and i-motif structure formation competes with duplex formation. In physiological salt conditions at 0.1 MPa, the duplex is formed by almost all strands. G4 forms preferentially in the presence of certain metal ions (e.g., K^+). Because the i-motif is less than that of duplex under physiological conditions, no i-motif is formed unless G4 formation releases the C-rich strand from the duplex.[6] The *P-T* diagram based on data reported here indicates that under high pressure, the i-motif becomes more stable than the duplex (Fig. 5). Furthermore, the destabilization of G4 at high pressure (> 200 MPa) results in the presence of the i-motif structure in the absence of G4. In the presence of a crowding agent, G4 is less destabilized by high pressure and the i-motif and G4 dominate the structures observed at high pressure.

We hypothesize nucleic acid functions can be triggered by pressure and temperature. That pressure and temperature exert an effect on protein function has been demonstrated. Thermostable RecA from *Thermus thermophilus*, which survives under pressure and a high temperature in deep sea thermal vents, forms a more stable complex with DNA under high pressure and temperature than does the RecA from *E.coli*, an organism that grows optimally at 37 °C under ambient pressure. The shapes of *P-T* phase diagrams from the *Thermus thermophilus* RecA and *E.coli* RecA differ significantly.[41] Pressure and temperature also affect individual domains within proteins. Folding of ubiquitin evaluated by NMR revealed that the *P-T* dependence of hydrogen bonds within the protein varied with position and whether or not the interaction was involved in the substrate recognition domain or maintenance of tertiary structure.[42] The pressure and temperature dependencies of canonical and non-canonical DNA structures reported here and in previous work suggest that nucleic acids, like proteins have pressure and/or temperature dependent functions in cells.[27] Temperature may impact the storage or transfer of genetic information because the duplex structure is perturbed by temperature but not pressure. Pressure may induce functions of non-canonical structures such as the i-motif and G4 as the stabilities of these two structures depend dramatically – and in opposite ways – on pressure. Pressure may have a regulatory effect on gene expression through effects on sequences with G4 and i-motif forming potential that are found in promoter regions of oncogenes and in genes involved in normal cellular function. G4s can be stabilized by binding of proteins such as Nucleolin, and, although no proteins that stabilize the i-motif structure have yet been identified, evidence suggests that transcription of the *BCL2* gene may be controlled by formation of an i-motif structure and this regulation may also be affected by pressure change.[45-46]

The pressures used in this study are much higher than those experienced by organisms on earth under atmospheric pressure. On prebiotic earth, however, pressure and temperature were likely much higher[47] and there are organisms that survive in the deep sea under more than 100 MPa of pressure.[48] Interestingly, our results suggest that DNA structural transitions occur at about 100 MPa (Figs. S11 and S13). This finding implies that pressure stimuli can impact DNA structure inside living cells. Pressure changes may have initiated expression of genetic information in protocells and gene expression may be control in deep sea organisms by pressure. Furthermore, there also reports that compartmentalized space results in a high pressure-like environment that facilitates molecular reactions.[49, 50] Therefore, even in modern living cells under atmospheric pressure, compartmentalization in cellular organelles may result in regions of high pressure. Cancer cells have lower water content than normal cells, and many microorganisms have slightly acidic cytosolic conditions that may stabilize i-motif structures. If gene regulation from i-motif structure exists without forming G4, which has not been reported yet, pressure changes give additional option to choose gene regulation of G4 type or i-motif type by controlling the formation of both G4 and i-motif.

Conclusions

In conclusion, we demonstrated that formation of the DNA i-motif structure was stabilized by high pressure. The stabilization effect on i-motif was also observed under molecular crowding conditions, whereas the destabilizing effect of pressure on G4 formation is moderated by the presence of a molecular crowding agent. *P-T* stability diagrams suggest that gene expression may thus be dynamically regulated as duplex is destabilized and the non-canonical structures stabilized as pressure is increased.

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