Human Telomeric DNA Forms Parallel-Stranded Intramolecular G-Quadruplex in K+ Solution under Molecular Crowding Condition

Yong Xue,[†] Zhong-yuan Kan,[†,§] Quan Wang,[†] Yuan Yao,[†] Jiang Liu,[†] Yu-hua Hao,[‡] and Zheng Tan*,[†,‡]

Contribution from the Laboratory of Biochemistry and Biophysics, College of Life Sciences, Wuhan University, Wuhan 430072, P. R. China, and State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P. R. China

Received May 1, 2007; E-mail: z.tan@ioz.ac.cn; tanclswu@public.wh.hb.cn

Abstract: The G-rich strand of human telomeric DNA can fold into a four-stranded structure called G-quadruplex and inhibit telomerase activity that is expressed in 85−90% tumor cells. For this reason, telomere quadruplex is emerging as a potential therapeutic target for cancer. Information on the structure of the quadruplex in the physiological environment is important for structure-based drug design targeting the quadruplex. Recent studies have raised significant controversy regarding the exact structure of the quadruplex formed by human telomeric DNA in a physiological relevant environment. Studies on the crystal prepared in K+ solution revealed a distinct propeller-shaped parallel-stranded conformation. However, many later works failed to confirm such structure in physiological K+ solution but rather led to the identification of a different hybrid-type mixed parallel/antiparallel quadruplex. Here we demonstrate that human telomere DNA adopts a parallel-stranded conformation in physiological K+ solution under molecular crowding conditions created by PEG. At the concentration of 40% (w/v), PEG induced complete structural conversion to a parallel-stranded G-quadruplex. We also show that the quadruplex formed under such a condition has unusual stability and significant negative impact on telomerase processivity. Since the environment inside cells is molecularly crowded, our results obtained under the cell mimicking condition suggest that the parallel-stranded quadruplex may be the more favored structure under physiological conditions, and drug design targeting the human telomeric quadruplex should take this into consideration.

Introduction

Tandem repetitive guanine-rich DNA can fold in the presence of K+ or Na+ into a four-stranded structure named G-quadruplex via Hoogsteen hydrogen bonding.[1] Quadruplexes formed by human telomeric DNA have become a focus of attention in recent years because of their role in important biological processes, such as aging and cancer,[2] and potential as a therapeutic target for cancer.[3,4] The intramolecular quadruplexes of human telomere can adopt a variety of folding topologies according to the relative orientations of the four strands held together by the G-quartets (Figure 1). Accurate information on the exact conformation that the human telomere quadruplex adopts under physiological condition is important not only for understanding its biological function but also for structure-based drug design to succeed.

Animal cells maintain high concentration of K+ (150 mM) and low Na+ (5−15 mM) intracellularly.[5] Recently, investigations on the structure of the intramolecular human telomere quadruplex in physiological K+ solution raised extreme controversy. The quadruplex in Na+ solution is well-characterized by NMR to adopt antiparallel-stranded conformation (Figure 1A,B).[6] The structure in K+ solution had not been as well-identified as that in Na+ solution and was suggested to contain both antiparallel and parallel arrangements.[7−10] In 2002, a fundamentally distinct propeller-like parallel-stranded structure (Figure 1C) was resolved for the crystal of the human telomere quadruplex made in K+ solution,[11] which evoked intense interest in exploring the structure of the human telomere quadruplex in noncrystalline state in physiological K+ solution. However, a large number of studies that followed reported results incon-

[†] Wuhan University.
[‡] Chinese Academy of Sciences.
[§] Present address: Department of Biochemistry and Biophysics, University of Pennsylvania, 422 Curie Boulevard, Philadelphia, PA 19104.
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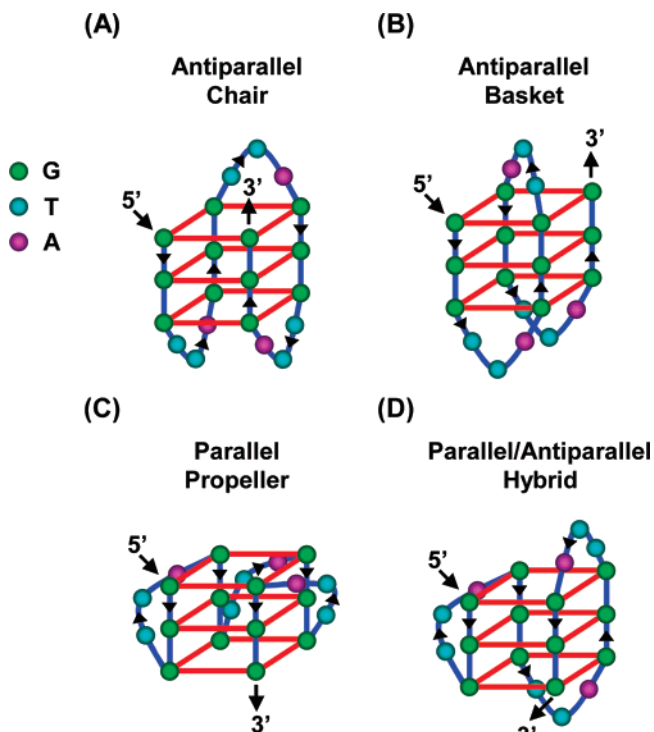(11) Parkinson, G. N.; Lee, M. P.; Neidle, S. *Nature* **2002**, *417*, 876−880.

Figure 1. Folding topologies of intramolecular human telomeric G-quadruplexes. In each structure, the four G triplet strands (straight lines) are held together by three stacked planar G-quartets (red rectangles) and connected by three TTA loops (curved lines). (A) Antiparallel chair type with three lateral loops. (B) Antiparallel basket with one diagonal and two lateral loops. (C) Parallel propeller with three external loops. (D) Parallel/antiparallel hybrid with one external and two lateral loops.

sistent with the crystal structure.[12−18] In 2006, three laboratories reported a new hybrid-type structure in K⁺ solution with mixed parallel/antiparallel strands within the quadruplex (Figure 1D), which was proposed as the favored conformation adopted under physiological condition.[19−21] It was suggested that the parallel-stranded crystal conformation might have been selected under the particular condition associated with the preparation of crystal and thus may not present the biologically relevant form of the human telomere quadruplex in K⁺ solution.[17]

In this work, we show that the intramolecular human telomere quadruplex adopts parallel-stranded conformation in the non-crystalline state in K⁺ solution under molecular crowding condition as the K⁺ crystal quadruplex does. Such a structure was supported by circular dichroism (CD), gel electrophoresis, and fluorescence analysis. Because the intracellular environment is molecularly crowded with macromolecules of up to 40% (w/v) in concentration,[22] we propose that such a conformation may

well represent the favored structure that the human telomere quadruplex adopts under physiological condition. Moreover, such a quadruplex was found to have dramatically enhanced stability. To exemplify its potential biological relevance, we show that the unusually stable parallel-stranded quadruplex greatly decreases telomerase processivity.

Materials and Methods

Oligonucleotides. Regular oligonucleotides were purchased from Sangon Biotech (Shanghai, China) or TaKaRa Biotech (Dalian, China). Oligonucleotides containing fluorescent analogue 2-aminopurine (Ap) were purchased from IDT (Coralville, IA). The concentration of oligonucleotide was determined from absorbance and extinction coefficient at 260 nm. The extinction coefficient for $G_3(T_2AG_3)_3$ (215 000 M^{-1} cm^{-1}) was calculated by the nearest-neighbor method as described[23] and that for the Ap-containing oligonucleotides (202 000 M^{-1} cm^{-1}) was provided by the manufacturer. Experiments were all carried out in buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM of salt indicated in the absence or presence of PEG 200. All samples were heated at 95 °C for 5 min and then slowly cooled to room temperature before analysis unless otherwise indicated.

Circular Dichroism Spectroscopy. CD spectra were collected from 320 to 220 nm on a Jasco J-810 spectropolarimeter using 1-nm bandwidth. Temperature was controlled using a digitized water bath integrated with the instrument.

Gel Electrophoresis. Native gel electrophoresis was run on 16% polyacrylamide gel containing 40% (w/v) PEG 200 and 150 mM KCl at 4 °C, 12.5 V/cm in TBE buffer containing 150 mM KCl. Oligonucleotides were 5′-end-labeled with [γ-³²P]ATP using T4 poly-nucleotide kinase (Fermentas, Vilnius, Lithuania). Denaturing gel electrophoresis was run on 19% polyacrylamide gel containing 7 M urea. Gels were autoradiographed with X-ray film.

Fluorescence Experiments. Measurements were carried out at 25 °C on a Spex Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Villeneuve d'Ascq, France). Emission spectra of oligonucleotides containing Ap were collected from 320 to 420 nm with excitation at 305 nm. For ascorbic acid quenching, Ap oligonucleotide solution was titrated with ascorbic acid stock solution, and fluorescence was measured at 370 nm with excitation at 305 nm.

Telomerase Processivity Assay. Telomerase extract was prepared from cultured HeLa cells as previously described[24] with an additional 40% ammonium sulfate precipitation. The pellet was resuspended to 5000 cells/μL of lysis buffer and frozen at −80 °C until use.

A modified version of the telomeric repeat amplification protocol assay specifically designed for analyzing telomerase processivity[25] was used as described with a TSNT internal standard (IS).[26] The extension reaction was performed using extract of 25 000 cells in the absence or presence of 40% (w/v) PEG 200. After extension, all samples were adjusted to contain same amount of PEG and subject to phenol-chloroform extraction and ethanol precipitation. Recovered oligonucleotide was redissolved in 20 μL of buffer, and 3 μL was used to provide templates for PCR amplification in a 30-μL volume containing 0.5 μM NT (5′-ATCGCTTCTCGGCCTTTT-3′) as the return primer for TSNT. The PCR products were resolved on 12% polyacrylamide gel, stained with ethidium bromide, and recorded on a ChemiImager 5500 (Alpha Innotech, San Leandro, CA). Telomerase activity was quantified as percent of the control, with the PCR efficiency calibrated against the internal standard.

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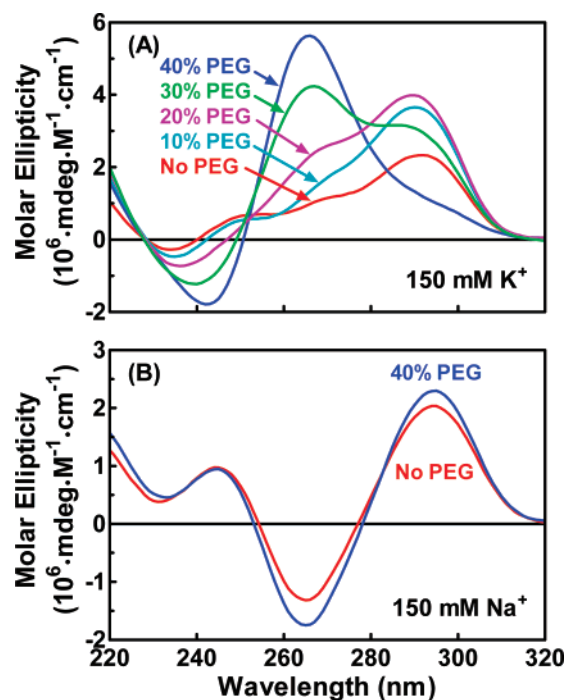(26) Kim, N. W.; Wu, F. *Nucleic Acids Res.* **1997**, *25*, 2595−2597.

Figure 2. Conformational conversion of the $G_3(T_2AG_3)_3$ quadruplex induced by PEG 200 assessed by CD spectroscopy. Quadruplex prepared in solution containing 150 mM (A) K^+ or (B) Na^+ in the absence or presence of PEG at the concentrations indicated.

Results

We used $G_3(T_2AG_3)_3$, the core sequence of the G-rich strand of human telomere DNA. Intracellular environment is characterized by high concentration of both K^+ (150 mM)[5] and macromolecules (up to 400 g/L).[22] To examine the structure of the human telomere quadruplex in a more physiologically relevant environment, CD spectroscopic analysis was conducted in K^+ and Na^+ solution in the presence of a most widely used crowding agent PEG 200 (Figure 2). The samples were heat-denatured and cooled to room temperature to ensure that the structures were formed under the intended environment before CD recording. Antiparallel structure is characterized by a negative peak near 260 nm and a positive peak near 295 nm, while parallel structure displays a negative peak near 240 nm followed by a positive peak near 265 nm.[7,27,28] In K^+ solution, the spectrum of $G_3(T_2AG_3)_3$ in the absence of PEG showed a small negative peak near 240 nm and a positive peak near 290 nm with a shoulder at 270 nm (Figure 2A), similar to those reported for the human telomere DNA in K^+ solution.[8–10,19,29] PEG induced a conformational conversion as manifested by the emerging of a positive peak at 265 nm and the diminishing of the peak at 290 nm with increase in PEG concentration. When PEG reached 40% (w/v), the peak at 290 nm disappeared, resulting in a spectrum similar to those of the parallel quadruplexes formed by many nonhuman telomere sequences.[7,30–38]

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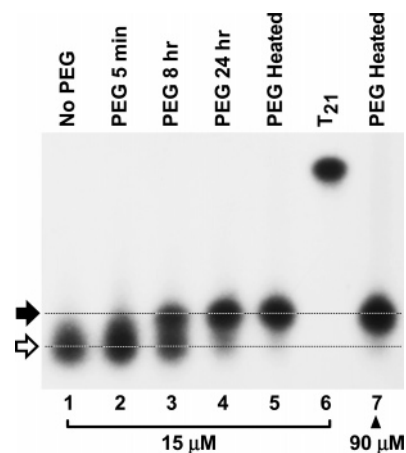
Figure 3. Conformational conversion of the $G_3(T_2AG_3)_3$ quadruplex induced by 40% (w/v) PEG 200 in 150 mM K^+ solution examined by gel electrophoresis. Samples of lanes 1–4 were prepared in K^+ solution containing no PEG with heat denaturation and renaturation. Those of lanes 2–4 were then incubated in the presence of PEG for the indicated period before electrophoreses. Samples of lanes 5–7 were prepared in K^+ solution in the presence of PEG, heat denatured, and renatured before electrophoresis. The emerging of the K^+/PEG quadruplex is indicated by the filled arrow and the disappearance of the K^+ quadruplex by the open arrow.

This fact suggests that $G_3(T_2AG_3)_3$ adopted a parallel-stranded topology in solution as in the K^+ crystalline state.[11] Even though exceptions have been reported where an antiparallel quadruplex formed by some sequences also showed a positive peak near 260 nm,[39–41] typical of a parallel quadruplex, it is very unlikely for the $G_3(T_2AG_3)_3$ to fall into this exception because the antiparallel structure of the human telomere quadruplex[6] is known to display a positive peak near 295 rather than 260 nm[7,8,19] as is shown in Figure 2B. PEG did not alter the conformation of the quadruplex in Na^+ solution, which agreed with previous studies where the effect of PEG on human telomere sequence was examined in Na^+ solution.[17,36]

The PEG-induced conformational conversion was further studied by gel electrophoresis (Figure 3). In this experiment, K^+ quadruplex samples were first prepared in K^+ solution containing no PEG with heat denaturation and renaturation, then PEG was added to 40% (w/v), and the mixtures was incubated for various periods of time without further heat denaturation and renaturation before electrophoresis. The conformational conversion, which took more than 24 h to complete, is clearly illustrated by the appearance of a new band (K^+/PEG quadruplex) and the disappearance of the old one (K^+ quadruplex). The slower migration of the K^+/PEG quadruplex relative to that of the K^+ quadruplex indicates that the parallel-stranded quadruplex is less compact than the hybrid-type K^+ quadruplex.

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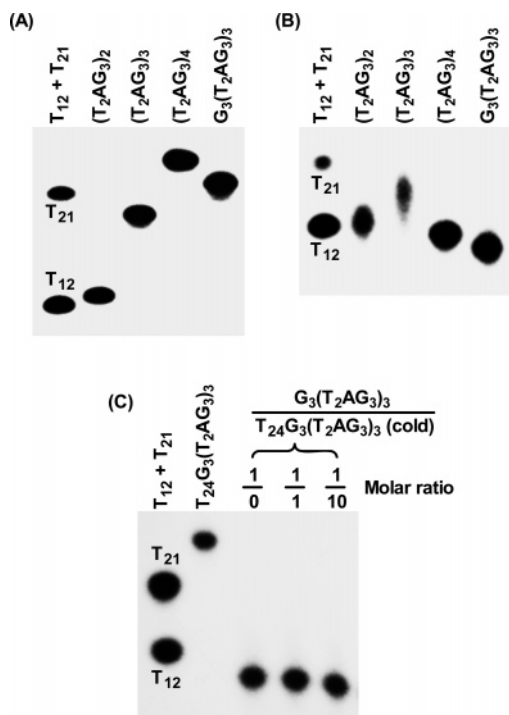
Figure 4. Gel electrophoretic analysis of intramolecular folding of $G_3(T_2$-$AG_3)_3$ in 150 mM K^+ solution containing 40% (w/v) PEG 200. (A) Denaturing gel. (B) Native gel. (C) Native gel showing the quadruplex formed by $G_3(T_2AG_3)_3$ in the presence of $T_{24}G_3(T_2AG_3)_3$. Samples in (A) were prepared and electrophoresed without added K^+ and PEG. Those in (B) and (C) were prepared and electrophoresed in the presence of K^+ and PEG.

Figure 5. Fluorescent emissions of 2-aminopurine (Ap) substitutes at different positions in the $G_3(T_2AG_3)_3$ quadruplex. Sample treatment before emission measurement: (A) heated at 95 °C for 5 min in the presence of 150 mM K^+, then cooled to 25 °C; (B) heated at 95 °C for 5 min in the presence of 150 mM K^+ and 40% (w/v) PEG 200, then cooled to 25 °C; and (C) heated at 95 °C for 5 min in the presence of 150 mM K^+, cooled to 25 °C, then PEG 200 was added to 40% (w/v) 5 min before the measurement.

In a previous study where experimentally determined and calculated sedimentation coefficients of human telomere quadruplex were compared, it was found that, for the quadruplex in Na^+ solution, the calculated value based on the NMR-determined structure was in excellent agreement with the experimentally determined value. In sharp contrast, the calculated value derived from the K^+ crystal structure was 24.9% smaller than the experimentally determined value for the structure in K^+ solution,[17] which also shows that the parallel-stranded quadruplex is less compact than the hybrid-type K^+ quadruplex. Therefore, the slower migration of the K^+/PEG quadruplex seen in our electrophoresis is in agreement with the smaller sedimentation coefficient of the K^+ crystal structure, providing additional support to the parallel-stranded conformation of the K^+/PEG quadruplex.

The slow conversion seen in the electrophoresis experiment demonstrates that heat denaturation and renaturation treatment is necessary in preparing samples such that the structures are formed in the intended environment. In a previous work by Li et al. where the sequence $AG_3(T_2AG_3)_3$ was used, PEG 400 was found to induce a development of a positive CD peak at 265 nm.[17] Such a structural conversion was incomplete in that the 290-nm peak did not decrease and the 265-nm peak was still lower than the 290-nm peak even at 1.4 M (56% w/v) of PEG. It seems that their samples were not heat-denatured to ensure that the structures were formed under the new condition with PEG. This prevented them from revealing the true conformation that forms in PEG-containing solution, but rather led to a conclusion against the parallel-stranded quadruplex.

Since PEG 200 has been reported to induce a transition from the intramolecular quadruplex to multimolecular G-wire in
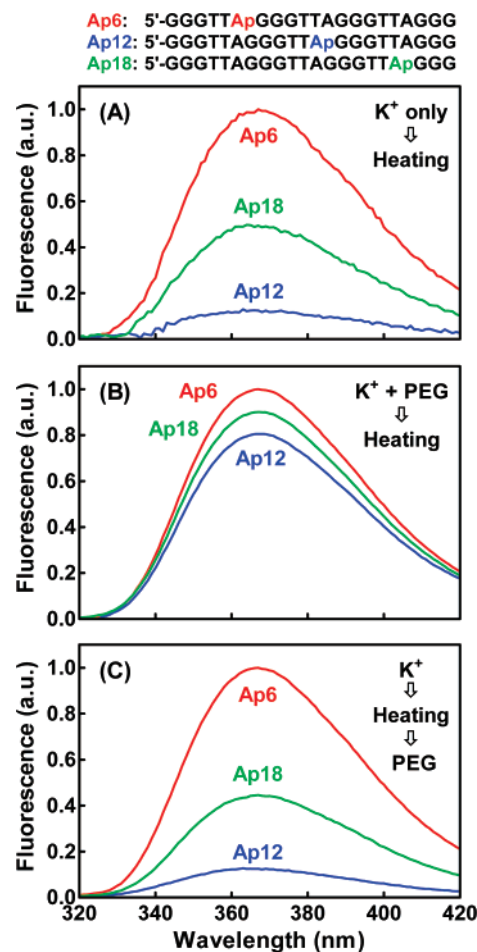
Tetrahymena telomere DNA $T_2(G_4T_2)_3G_2$,[36] the stoichiometry of the human K^+/PEG quadruplex was analyzed by gel electrophoresis (Figure 4). Folding into the intramolecular quadruplex results in increased mobility of oligonucleotide in gel electrophoresis.[8,42] The much faster migration of the K^+/PEG quadruplex relative to that of the length-matched 21mer poly-T (Figure 3) suggests that the K^+/PEG quadruplex was an intramolecular structure. In Figure 4A,B, the mobility of $G_3(T_2AG_3)_3$ was compared with several reference oligonucleotides. Without forming a secondary structure, the $G_3(T_2AG_3)_3$ and $(T_2AG_3)_n$ ($n = 2, 3, 4$) in denaturing gel displayed mobilities inversely proportional to their sizes (Figure 4A) as previously reported.[29,42] Formation of the K^+/PEG quadruplex by $G_3(T_2$-$AG_3)_3$ and $(T_2AG_3)_4$ resulted in increased migrations that were even faster than that of the 12mer poly-T in native gel (Figure 4B), which can be interpreted as intramolecular quadruplex formation by the two oligonucleotides.[8] To further exclude the possibility of intermolecular quadruplex formation, $G_3(T_2AG_3)_3$ was mixed with $T_{24}G_3(T_2AG_3)_3$ to prepare the quadruplex. $G_3(T_2AG_3)_3$ carrying a nonquadruplex-forming sequence at the 5′ end forms a quadruplex with similar CD characteristics as

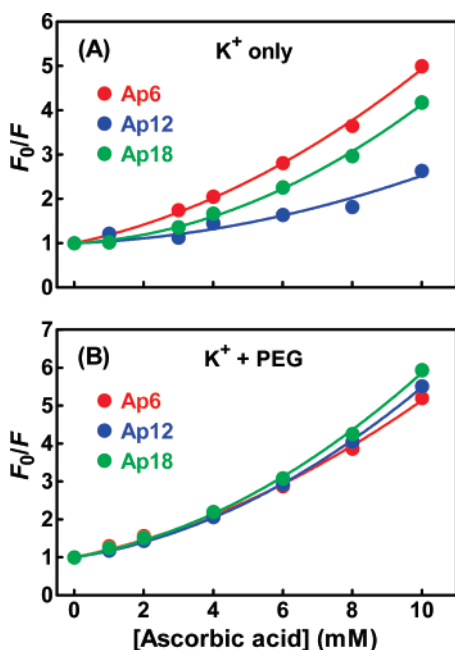(42) Murchie, A. I.; Lilley, D. M. *EMBO J.* **1994**, *13*, 993−1001.

Figure 6. Accessibilities of 2-aminopurine (Ap) substitutes at different positions in the $G_3(T_2AG_3)_3$ quadruplex in solution containing (A) 150 mM K^+ and (B) 150 mM K^+ and 40% (w/v) PEG 200 assessed by ascorbic acid quenching. F_0 and F are the fluorescence intensities of Ap in the absence and presence of ascorbic acid, respectively.

Figure 7. CD-melting profiles of the $G_3(T_2AG_3)_3$ quadruplex in 150 mM (A) K^+ or (B) Na^+ solution in the absence and presence of 40% (w/v) PEG 200. Denaturation was monitored at (A) 265 nm (with PEG) or 290 nm (without PEG) and (B) 295 nm while temperature was increased from 20 to 80 °C at 1.0 °C/min. The numbers by the curves are the melting temperature (T_m) values of the corresponding curves.

$G_3(T_2AG_3)_3$.[43] If the two oligonucleotides could form a multimeric intermolecular quadruplex, hybrid quadruplexes of intermediate sizes would be expected in the mixture of them.[44,45] The gel in Figure 4C shows that this was not the case.

The parallel-stranded intramolecular quadruplex predicts structural symmetry for the three external TTA loops (Figure 1C).[11] To further verify the parallel-stranded conformation of the K^+/PEG quadruplex, studies were carried out using $G_3(T_2AG_3)_3$ with 2-aminopurine (Ap) substitutions at different adenine residues (Figure 5, Ap6, Ap12, Ap18). Ap is a fluorescent adenine isomer that has been widely used to probe local conformational changes in DNA.[46−48] Specifically, substitution of adenine in quadruplex with Ap has been shown to provide a sensitive tool for probing loop environment without altering overall quadruplex conformation.[17,49,50] In our study, the fluorescence intensities of the Ap's in the K^+ quadruplex followed the order Ap6 > Ap18 > Ap12 (Figure 5A) as previously reported for the same structure.[17] The large differences in fluorescence emission between the Ap's reflected the heterogeneity of the TTA looping conformations in the K^+ quadruplex. In contrast, the fluorescence intensity of the Ap's in the K^+/PEG quadruplex became very similar to each other
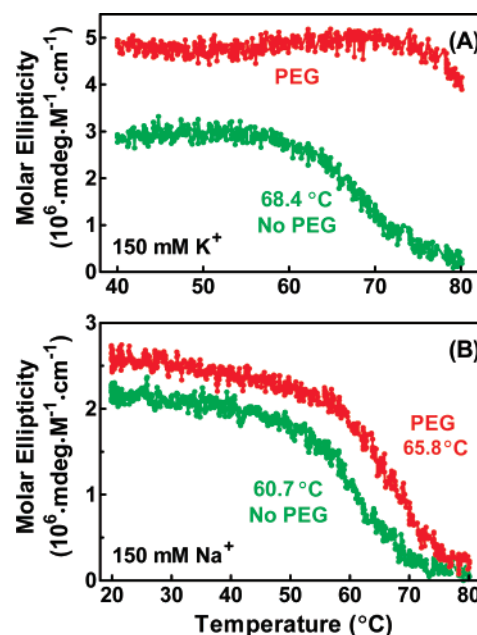
(Figure 5B), which agrees with the predicted structural symmetry (Figure 1C) that should provide similar local environment for the adenine residues. This result was not a direct effect of PEG on Ap fluorescence but reflected the conformation conversion induced by PEG. This is because when PEG was added to the K^+ quadruplex and the sample was measured within several minutes without heat denaturation and renaturation, the order and relative magnitude of fluorescence intensity of the three Ap's showed little change (Figure 5C vs 5A). In this case, the quadruplex remained in the K^+ conformation within the short period in the presence of PEG because of the extremely slow conformational conversion (Figure 3). The environment of Ap's was also assessed by fluorescence quenching with ascorbic acid that has been used to probe solvent accessibility of Ap in the quadruplex.[49] While the Ap's in the K^+ quadruplex showed a large difference in accessibility (Figure 6A), those in the K^+/PEG quadruplex were almost identical (Figure 6B), further supporting the parallel-stranded topology in the K^+/PEG quadruplex (Figure 1C). The Ap6 in the K^+ quadruplex is hosted in an external loop according to the recently identified conformation,[19−21] (Figure 1D) and its higher extent of fluorescence quenching (Figure 6A) indicates that it was more accessible than the other two Ap's in the lateral loops. The conversion of the two lateral loops into external by PEG was accompanied by increased quenching for the two Ap's (Figure 6B).

The quadruplex inhibits telomerase activity in vitro and has been suggested to act as a negative regulator of telomere elongation in vivo.[51,52] It can be conceived that the inhibition will depend on the stability of the quadruplex. Stabilization of the quadruplex by many small molecules has been shown to

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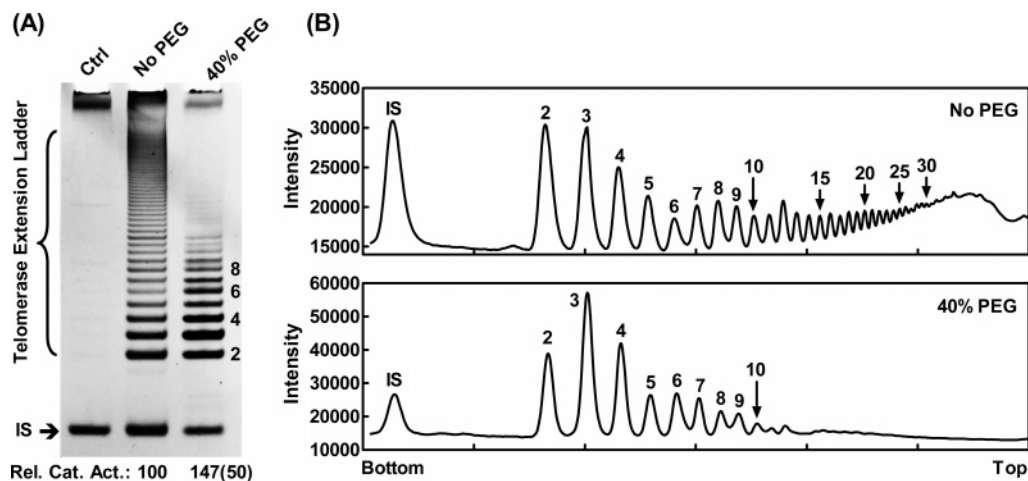
Figure 8. Effect of PEG on telomerase processivity and overall catalytic activity. Assays were carried out in the absence or presence of 40% (w/v) PEG 200. Extension products were amplified by PCR and resolved by gel electrophoresis. (A) Representative gel showing ladders of telomerase extension products. Telomerase processivity is reflected by the sizes of the extension products. Ctrl indicates assay using heat-inactivated telomerase extract and IS the band of internal standard. The numbers below the lanes are the mean relative catalytic activity of three experiments with standard deviation (in parentheses). The final concentration of K^+ in the assays was 63 mM. (B) Profiles of telomerase extension obtained by digital scan of the gel. Numbers above the peaks indicate the number of TTAGGG repeats added.

inhibit telomerase activity.[53] Since the parallel-stranded human telomere quadruplex has not been reported in solution state before, we analyzed the thermal stability of the quadruplex formed by $G_3(T_2AG_3)_3$ in the presence and absence of PEG by CD melting. The results in Figure 7A show that the K^+/PEG quadruplex had an unusual stability with a melting temperature (T_m) much greater than 80 °C. In contrast, the K^+ quadruplex had a T_m of only 68.4 °C. The stabilization induced by PEG in K^+ solution far exceeded that by PEG in Na^+ solution where the T_m increased by only 5.1 °C (Figure 7B) and those in a recent work in which PEG was reported to increase the T_m of the quadruplex formed by the thrombin DNA aptamer $G_2T_2G_2$-$TGTG_2T_2G_2$ by 4.6 °C in K^+ and 12.5 °C in Na^+ solution.[54] For the thrombin DNA aptamer, the stabilization of the quadruplex by PEG was attributed to the altered dehydration of DNA and cations and no structural conversion was observed for the quadruplex in either K^+ or Na^+ solution.[54] Thus, the structural conversion may have contributed to the much greater stabilization by PEG in K^+ solution seen in our study in addition to dehydration.

Given such unusual stability of the K^+/PEG quadruplex, we further examined how it would affect telomere elongation by telomerase. We used a PCR-based method that was specifically designed for analyzing telomerase processivity (the ability to continuously add telomere repeats without leaving the substrate).[25] We included in the assay an internal standard so that the catalytic activity (the amount of overall telomere repeat addition) could also be assessed.[26] The results in Figure 8 show that molecular crowding significantly depressed telomerase processivity as judged from the sizes of the extension products. In the absence of PEG, extension of up to 30 TTAGGG repeats was detectable on the gel. Longer products were present but not resolved well. In contrast, obvious extension was limited to about 10 repeats in the presence of PEG, but the band intensity of smaller products was elevated. PEG enhanced the

overall catalytic activity of the telomerase, resulting in an increase in the integrated band intensity compared to that in the assay without PEG. Therefore, the decreased processivity with PEG is explained by the increased stability of the quadruplex. In these assays, a nontelomeric sequence was used as substrate in large excess of telomerase. Since quadruplex formation requires four TTAGGG repeats, it is expected that quadruplex stabilization by molecular crowding would take effect when four or more repeats were added to the substrate. This would lead to an increased dissociation of telomerase from the substrate; however, the enzymes seemed efficiently translocated to other substrates to continue catalysis. For the large amount of input substrates containing no quadruplex, their extension might be more effective, and as a result lead to the increased overall catalytic activity.

Discussion

In this work, we observed that the reported parallel-stranded conformation adopted in the crystalline state was also adopted in the solution state under molecular crowding condition in K^+ solution by the human telomere intramolecular quadruplex. Parallel-stranded structure has been found in nonhuman telomere quadruplexes mostly in intermolecular form[7,30−38,55] and only in multimeric intermolecular quadruplexes formed by human telomere DNA of less than four repeats[14,56−58] before the first report of the parallel-stranded crystal conformation by Neidle's group.[11] Knowing the correct structure that human telomere quadruplex adopts under physiologically relevant condition is important not only for a better understanding of its biological implication, but also for successful drug designing. Because of the molecular crowding nature of the intracellular environment, our work suggests that the parallel-stranded conformation may be the more favored structure in a biologically relevant

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environment than the other types of quadruplexes.[20] This information should be useful for drug design targeting the human telomere quadruplex. The properties associated with the parallel-stranded structure may also have biological implications, as exemplified by the restriction on telomerase processivity.

Quadruplex conformation is sequence-dependent and can be affected by environmental factors. Molecular crowding has been reported to induce transition from antiparallel to parallel G-quadruplex in *Oxytricha nova* telomeric DNA in Na^+ solution[31] and transition from intramolecular G-quadruplex to long multistranded G-wire in *Tetrahymena* $(T_2G_4)_3T_2G_2$ but not in human $G_3(T_2AG_3)_3$ telomeric DNA.[36] The controversy about the physiological conformation of the human telomere quadruplex apparently arose from the different experimental conditions used in previous studies. While the majority of previous studies were carried out in dilute solution, we have shown that molecular crowding can induce quadruplex formation under salt-deficient condition.[29] This finding demonstrates that molecular crowding has a fundamental influence on the quadruplex since it has long been accepted that quadruplex formation requires metal ions. The conformational conversion induced by PEG observed in this work further demonstrates the impact of the effect of molecular crowding and emphasizes the importance of studying the human telomere quadruplex under physiologically relevant conditions. Under true intracellular condition, the presence of proteins may also affect the formation and conformation of the quadruplex. Whether telomere DNA can form a quadruplex and what conformation it adopts under such condition still awaits more investigations.