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Product analysis of photooxidation in isolated quadruplex DNA; 8-oxo-7,8-dihydroguanine and its oxidation product at 3′-G are formed instead of 2,5-diamino-4*H*-imidazol-4-one†

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The formation of quadruplex structure changed the site reactivity and the kinds of guanine photooxidation products of d(TGGGGT). In quadruplex DNA, 8-oxo-7,8-dihydroguanine (8oxoG) and dehydroguanidinohydantoin (Ghox) were mainly formed, although 2,5-diamino-4*H*-imidazol-4-one (Iz) was mainly formed in single-stranded DNA. In addition, 3′-guanine was specifically oxidized in quadruplex DNA compared with single-stranded DNA, which depended on the localization of the HOMO.

DNA bases are the key components for preservation of genetic information. However, DNA nucleobases are frequently oxidized *via* reactions with environmental agents such as UVA-light. Among the four DNA bases, guanine is the most easily oxidized. Additionally, guanines in contiguous guanine sequences, such as GG and GGG, in double-stranded DNA have lower redox potentials than single guanine sequences.[1] Thus, some guanine-rich sequences could be easily oxidized in living organisms. Guanine-rich sequences exist in many important regions, such as telomeres[2] and the promoter element of the proto-oncogene c-myc[3] in the human genome, and these sequences can form quadruplex DNA with eight Hoogsteen hydrogen bonds of four guanines. In recent computational investigations,[4] the quantity of quadruplex-forming sequences in the genome is over 3.5×10^5. The differences of DNA structure between single-stranded and quadruplex DNA may influence the process of oxidation. In this study, quadruplex DNA was isolated by HPLC, and the oxidation of the isolated quadruplex DNA was compared with that of the single-stranded DNA.

In particular, we attempt to directly analyze the oligomers containing the oxidation product. This approach enabled us to identify the kinds, the yields, and the locations of the oxidation products at once, unlike a recent report.[5] Since the shorter oligomer was appropriate for isolation and identification, we used the 6-mer DNA d(TGGGGT), which is the shortest oligomer among the quadruplex-formative sequences in the presence of several ions.[6] This 6-mer oligomer is the truncated telomeric sequence from Tetrahymena or Oxytricha.

The quadruplex structure of d(TGGGGT) was formed in KCl solution in this study, since the intracellular potassium ion concentration is high (approximately 140 mM). Since, d(TGGGGT) can formed in 1 mM KCl. The d(TGGGGT) in 10 and 0.1 mM KCl were heated to 80 °C, and incubated at 4 °C for 1 min, and the structures were determined by CD spectroscopy. The increasing typical CD spectrum around 260 nm of the quadruplex DNA was detected in 10 mM KCl (Fig. 1a).[7] In contrast, the CD spectrum around 260 nm was not observed in 0.1 mM KCl (Fig. 1b), and then the quadruplex DNA was not formed. Next, the d(TGGGGT) in 10 and 0.1 mM KCl were analyzed using HPLC, and the profiles are shown in Fig. 1c and d, respectively. In these profiles, two peaks at 8.2 and 18.0 min were detected in 10 mM KCl. The peaks were isolated using HPLC. Furthermore, the isolated products at 8.2 and 18.0 min in Fig. 1c were identified as a single-stranded DNA and a quadruplex DNA containing three potassium ions ($[C_{240}H_{297}N_{96}O_{144}P_{20}K_3]$ *m*/*z* 1889.80689, calculated for [M − 4H] 1889.80493) by electrospray ionization-mass spectrometry (ESI-MS) in negative-ion mode (Fig. S1, ESI†). Even though the isolated quadruplex DNA in 10 mM KCl at 4 °C was left for at least 3 days, single-stranded DNA was detected in less than 4% (Fig. 2). Although previous reports suggested that single-stranded DNA and quadruplex DNA were separable with HPLC,[8] isolation of quadruplex DNA was not described in the article. To our knowledge, isolation of quadruplex DNA was accomplished by using HPLC for the first time. Using this isolated quadruplex structures can eliminate the possibility of formation of oxidation products from single-stranded or double-stranded DNA.

Isolated quadruplex DNA (700 μM, DNA concentration was expressed as single-stranded molarity) in 10 mM KCl solution or

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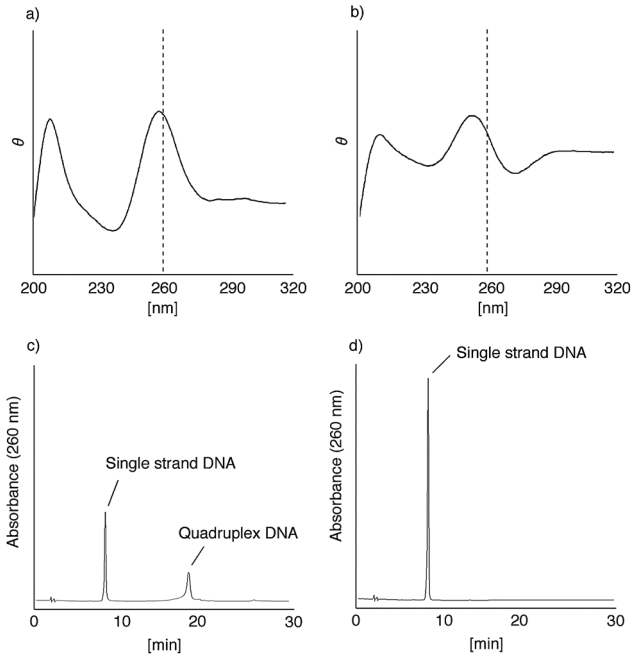


Fig. 1 The CD and HPLC profiles of d(TGGGGT) in KCl. CD spectroscopy was performed in (a) 10 mM or (b) 0.1 mM KCl. HPLC analyses were performed in (c) 10 mM KCl or (d) 0.1 mM KCl. Samples were analyzed by HPLC with a COSMOSIL $5C_{18}$-MS column (Nacalai Tesque, 5 μm, 150 × 4.6 mm, elution with a solvent mixture of 50 mM TEAA (pH 7), 5–30% CH_3CN/30 min at a flow rate of 1.0 ml min^{-1}) and monitored at 260 nm absorbance in panel c and d.

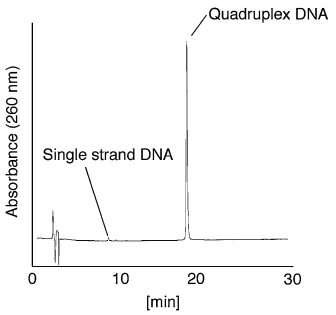


Fig. 2 HPLC analysis of isolated quadruplex DNA. Samples were analyzed by HPLC with a COSMOSIL $5C_{18}$-MS column (Nacalai Tesque, 5 μm, 150 × 4.6 mm, elution with a solvent mixture of 50 mM TEAA (pH 7), 5–30% CH_3CN/30 min at a flow rate of 1.0 ml min^{-1}) and monitored at 260 nm absorbance.

the single-stranded DNA in 0.1 mM KCl were photooxidized with 75 μM riboflavin (RF, *i.e.*, vitamin B_2), and these products were analyzed using HPLC.[9] To determine the oxidation products of single-stranded and quadruplex at almost the same conversion rate (approximately 20%),[10] we determined the rates of the oxidation in single-stranded and quadruplex DNA. The reaction of quadruplex DNA was analyzed after UVA irradiation for 0, 10, 20, and 30 min, and the reaction of single-stranded DNA for 0, 1, 2, and 3 min. The conversions of the single-stranded DNA and the quadruplex DNA after UVA irradiation are shown in Fig. S2 (ESI†). Those conversions were determined with HPLC. As a result, the quadruplex DNA was oxidized more slowly than the single-stranded DNA. Some groups reported that quadruplex of guanine-rich DNA strands reduced the rates of reactions,[11] which agreed with our results.

HPLC chromatograms following oxidation of single-stranded and quadruplex DNA were shown in Fig. 3. The single-stranded and quadruplex DNA were irradiated for 2 min and 20 min, respectively, and both conversion rates were approximately 20%. Those photooxidized solutions were analyzed by HPLC. In irradiated single-stranded DNA, three major peaks were detected at 20.5, 20.7, and 21.1 min, as shown in Fig. 3b. These products were isolated and identified as oligomers containing 2,5-diamino-4*H*-imidazol-4-one (Iz) ($[C_{58}H_{74}N_{23}O_{36}P_5]$ *m/z* 910.66052, calculated for [M − 2H] 910.65937) by ESI-MS in negative-ion mode (Fig. 3a and S3†). The location of Iz in the oligomer was identified by piperidine treatment as previously reported.[12] The peaks at 20.5 and 20.7 min were identified as d(TGGGIzT) and d(TIzGGGT), respectively (Fig. S4a–c, ESI†). The peak at 21.1 min was composed of two products: d(TGIzGGT) and d(TGGIzGT).

The HPLC profiles of oxidized guanine in quadruplex DNA significantly differed from those in single-stranded DNA.[13] In irradiated quadruplex DNA, two major peaks were detected at 18.6 and 20.4 min (Fig. 3c). The peaks at 18.6 and 20.4 min in Fig. 3c were isolated and identified as the oligomer containing dehydroguanidinohydantoin (Ghox) ($[C_{59}H_{75}N_{24}O_{37}P_5]$ *m/z*

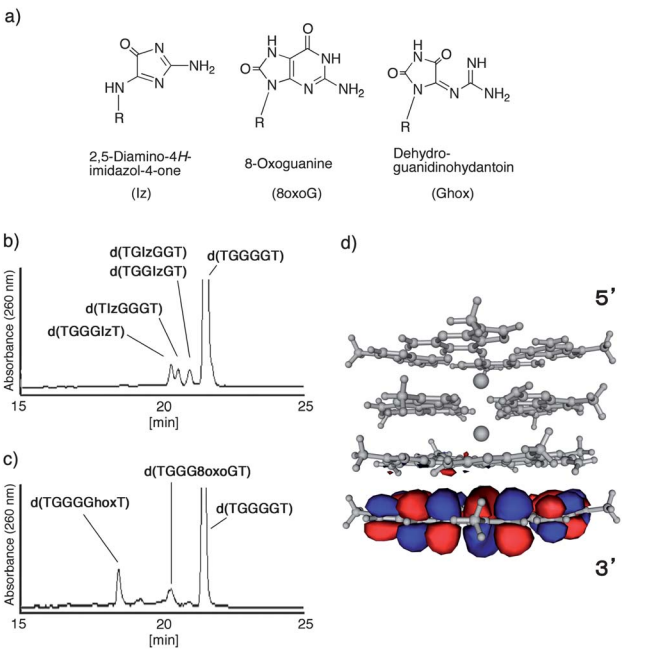


Fig. 3 Analysis of photooxidation products in quadruplex and single-stranded DNA. (a) Structures of guanine photooxidation products. (b) Single-stranded d(TGGGGT) (700 μM) with 75 μM RF in 0.1 mM KCl was irradiated at 365 nm for 2 min. (c) Quadruplex d(TGGGGT) (700 μM) with 75 μM RF in 10 mM KCl and 5 mM cacodylate buffer (pH 7) was irradiated at 365 nm for 20 min. Samples were analyzed by HPLC with a CHEMCOBOND 5-ODS-H column (Chemcopak, 5 μm, 150 × 4.6 mm, elution with a solvent mixture of 50 mM NH_4OAc (pH 7), 0–10% during 0–30 min, 10–30% during 30–35 min, CH_3CN at a flow rate of 1.0 ml min^{-1}) and monitored at 260 nm absorbance. (d) The localization of the HOMO in quadruplex DNA.

932.16758, calculated for [M − 2H] 932.16228) and the oligomer containing 8-oxo-7,8-dihydroguanine (8oxoG) ([$C_{60}H_{75}N_{24}O_{37}P_5$] *m/z* 938.16411, calculated for [M − 2H] 938.16228), respectively, by ESI-MS in negative-ion mode (Fig. 3a and S5, ESI†). The unoxidized single-stranded DNA was detected at 21.7 min (Fig. 3c). Although photooxidation in single-stranded DNA mainly produced the oligomers containing Iz, they were not detected in quadruplex DNA. The location of Ghox was then identified by piperidine treatment.[14] As a result, d(TGGGGhoxT) was detected (Fig. S4d, ESI†). Meanwhile, 8oxoG was stable under conditions of piperidine treatment.[15] Therefore, four synthetic oligomers containing 8oxoG and its oxidation products were analyzed by HPLC, and the peak at 20.4 min in Fig. 3c was confirmed by those HPLC profiles (Fig. S6, ESI†). As a result, the peak at 20.4 min was identified as d(TGGG8oxoGT). Surprisingly, the experiments shown in Fig. 3 revealed that the 3′-guanine of d(TGGGGT) was selectively oxidized in quadruplex DNA. In double-stranded DNA, 3′-guanine was hardly oxidized by the photooxidation of 5′-GG-3′,[1] 5′-GGG-3′,[16] and 5′-GGGG-3′.[17] To the best of our knowledge, specific oxidation of 3′-guanine in guanine-rich sequences has not previously been reported.

Thus, the guanine oxidation products from quadruplex DNA differ from those from single-stranded DNA, with differences in the structure possibly leading to alterations in the oxidation pathway of guanine. The one-electron oxidation[18] and the oxidation with singlet oxygen[19] were mediated by RF under UVA irradiation. To determine whether singlet oxygen was involved in the oxidation of guanine in single-stranded or quadruplex DNA, single-stranded or quadruplex DNA was photooxidized in 80% D_2O, the solvent in which singlet oxygen is known to have a longer half-life. As a result, the amounts of these oxidation products were little changed by 80% D_2O (Fig. 4). Thus, the oxidations were likely to undergo one-electron oxidation in single-stranded and quadruplex DNA, and singlet oxygen was not involved in the reactions.

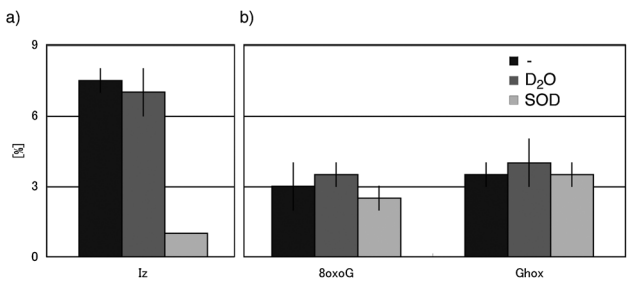
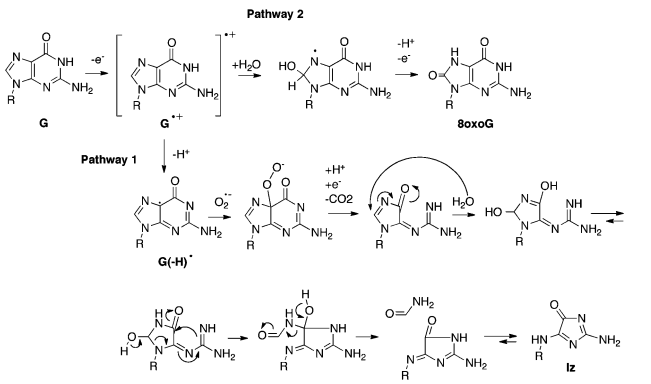


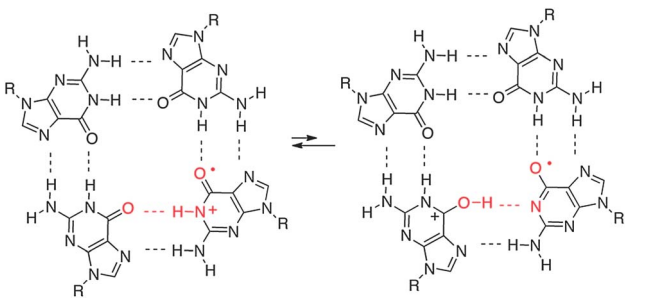
Fig. 4 The oxidations with SOD or D_2O. Single-stranded d(TGGGGT) (700 μM) with 75 μM RF in 0.1 mM KCl and 5 mM cacodylate buffer (pH 7) was irradiated at 365 nm for 2 min. Quadruplex d(TGGGGT) (700 μM) with 75 μM RF in 10 mM KCl and 5 mM cacodylate buffer (pH 7) was irradiated at 365 nm for 20 min. The reactions were initially performed in 80% D_2O or with 20 U μl^{-1} SOD. The amounts of oxidation products were calculated using HPLC. The mean values and errors were calculated using data from two independent experiments, and the error for Iz was not detected in the reaction with SOD. (a) Amounts of oligomers containing Iz in irradiated single-stranded DNA. (b) Amounts of oligomers containing 8oxoG or Ghox in irradiated quadruplex DNA.

One-electron oxidation of guanine generates a guanine radical cation ($G^{\cdot+}$),[20] and $G^{\cdot+}$ undergoes two competitive pathways (Scheme 1). In pathway 1, $G^{\cdot+}$ deprotonates at the N1 position to produce the neutral guanine radical [G(–H)˙], and the addition of superoxide ($O_2^{\cdot-}$)[21] to G(–H)˙ produces a transient peroxyl radical. Subsequently, nucleophilic addition of water induces the subsequent rearrangement and release of formamide,[21a] and leads to the formation of Iz. In pathway 2, hydration of $G^{\cdot+}$ is caused. Following the generation of 8-hydroxy-7,8-dihydroguanyl radical,[22] the radical is oxidized to 8oxoG (see ESI†). Since the major oxidation product of 8oxoG by one-electron oxidation was Ghox,[23] the d(TGGGGhoxT) should be formed by d(TGGG8oxoGT). In quadruplex DNA, hydrogen bond between the N1 proton of $G^{\cdot+}$ and the O6 of its neighbor guanine is formed (Scheme 2). Therefore, N1 proton in quadruplex DNA is more strongly retained than N1 proton in single-stranded DNA (see ESI†). Pathway 1 was blocked by the inhibition of deprotonation in the quadruplex, and 8oxoG was considered to be mainly formed in quadruplex DNA.

The 3′-guanine of d(TGGGGT) sequences was selectively oxidized in quadruplex DNA (Fig. 3c). Then, we calculated localization of the highest occupied molecular orbital (HOMO) of the quadruplex structure (see ESI†). As a result, the estimated HOMO was localized on the 3′-guanine of d(TGGGGT) (Fig. 3d), and the calculated result was matched with the experimental results (Fig. 3b and c). We concluded that the specific oxidation of 3′-guanine was induced by the localization of the HOMO.



Scheme 1 The proposed pathways for the oxidation of guanine in quadruplex DNA.



Scheme 2 The proton shift in $G^{\cdot+}$/G base pair in quadruplex DNA.

In summary, isolation and identification of quadruplex DNA were accomplished by using HPLC and ESI-MS (Fig. 2 and S1†). We attempted to identify the oxidation products of this isolated quadruplex and single-stranded DNA. In quadruplex DNA, major photooxidation products of guanine were identified as 8oxoG and its oxidation product, Ghox (Fig. 3c). The single-stranded DNA and quadruplex DNA significantly differed in the kinds of major oxidation products. Furthermore, 3′-guanine was mainly oxidized in quadruplex. We show that the specific oxidation of 3′-guanine depended on the localization of the HOMO (Fig. 3d). We found that the kinds and the locations of guanine oxidation products depended on the structures of quadruplex or single-stranded DNA.

Many important guanine-rich oligonucleotides can form quadruplex structure, and the quadruplex stability influences the telomere elongation by telomerase[24] and the transcription activity of c-myc.[25] 8oxoG affects the stability of quadruplex,[26] but it is not investigated whether the other oxidation products except for 8oxoG affect the quadruplex stability. Therefore, we will investigate the relation between this specific oxidation and the stability of quadruplex DNA.

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