Studies on the One-Electron Oxidation of DNA and DNA Alkylation

Takashi Nakamura

2000
Preface

The study presented in this thesis has been carried out under the direction of Professor Isao Saito at the Department of Synthetic Chemistry and Biological Chemistry of Kyoto University during April 1994 to March 2000. The study is concerned with studies on the one-electron oxidation of DNA and DNA alkylation.

The author wishes to express his sincere gratitude to Professor Isao Saito for his kind guidance, valuable suggestions, and encouragement throughout this work. The author is deeply grateful to Associate Professor Kazuhiko Nakatani for his valuable suggestions, and encouragement throughout this work. The author is also indebted to Professor Hiroshi Sugiyama, Assistant Professors Kenzo Fujimoto, Akimitsu Okamoto, Hisafumi Ikeda and Kiyohiko Kawai for their helpful suggestions and discussions. The author is also indebted to Professors Shosuke Kawanishi, Kizashi Yamaguchi, Associate Professor Yasunori Yoshioka, Assistant Professor Takashi Morii for their helpful discussions.

The author is grateful to the Supercomputer Laboratory, Institute for Chemical Research, Kyoto University for providing computation time. The author is also grateful to Kyoto University Radioisotope Research Center for providing the space for $^{32}$P-end-labeled DNA cleavage experiments. The author is grateful to Mr. Haruo Fujita and Mr. Tadao Kobatake for the measurements of NMR spectra and mass spectra, respectively. The author is thankful to Professor Junzo Sunamoto and Associate Professor Kazunari Akiyoshi for the measurements of circular dichroism. The author wishes to thank Dr. Masami Takayama and Mr. Kohzo Yoshida for their collaboration. The author is grateful to Messrs. Junya Shirai, Yasuki Komeda, Kazuhiro Tanabe, Shuji Ikeda, Nobuhiro Higashida, Kazuhiro Fujisawa, Shinsuke Sando, Shigeo Matsuda, Katsuhito Kino, Chikara Dohno, Takahiro Matsuno, Shinya Hagiwara, Kaoru Adachi, Toshiji Taiji, Takashi Yoshida, Hiroyuki Kumazawa and Kazuo Tanaka for their helpful suggestions and hearty encouragement.
The author is thankful to Messrs. Yutaka Ikeda, Naoki Ogawa, Masayuki Hayashi, Yohei Ozeki, Shigenori Ishihara, Makoto Ozeki, Taisuke Iwanami, Satoshi Okuda, Hiroshi Miyazaki, Yusuke Nomura, Mitsuhiro Iwasaki and Dr. Heike Heckroth for their helpful suggestions and hearty encouragement. The author also thanks to other members of Prof. Saito's research group and all the people supporting him.

Finally, the author expresses his deep appreciation to his parents, Mr. Yoshiaki Nakamura and Mrs. Sumie Nakamura for their constant assistance and affectionate encouragement.

Takashi Nakamura
January, 2000

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General Introduction

Were it not for the efficiency of DNA repair enzymes, oxidative damage to nucleic acids would be life-threatening if not lethal. It has been estimated that one in 40,000 guanines in the genome are present as 7,8-dihydro-8-oxo-guanine (8-oxo-G) under normal conditions; thus, in excess of 30,000 8-oxo-Gs may be present at any given time in a human cell. The formation and subsequent chemistry of this oxidized nucleobases are therefore of particular interest to human health. Furthermore, oxidation of nucleic acids is used as a tool both in medicine and in molecular biology. In the former area, nucleic acid-directed drugs such as Fe-bleomycin, ene-diynes, and quinone antibiotics cause oxidative damage to DNA. In molecular biology, oxidative reagents that react predictably with nucleic acids can provide information about their three-dimensional, folded structures.

The electron-rich nucleobases are prime targets for reaction with electrophiles including oxidizing agents. Guanine, with the lowest oxidation potential \( E^0 = 1.29 \text{ V vs. NHE} \), is the most subject to oxidation by a one-electron mechanism and is also highly reactive toward other oxidants including singlet oxygen and oxyl radicals (HO', RO', etc.). For example, photosensitized irradiation of DNA leads to a complex array of reactions, but much of the chemistry is centered on the guanine. An overall scheme for photosensitized DNA modification is shown in Figure 1.

![Figure 1. Chemistry resulting from photosensitized irradiation of DNA (Adopted from ref 13.)](image-url)
Guanine oxidation products can arise from a variety of pathways including type I reactions that involve electron transfer from G to a photoexcited-state oxidant, type II reactions involving singlet oxygen production, or the generation of other oxidants including halogen free radicals (X·), oxidized photosensitizer (S'), or secondary species derived from oxyl radicals (O₂·-, HO·). A large number of photosensitizers have been studied with DNA and RNA. For examples, the compounds that induce 5'->G-3' selective cleavage by photoirradiation and piperidine treatment are shown in Figure 2.

As early as 1985 it was observed that the reactivity of guanines toward oxidants including ionizing radiation was sensitive to sequence. Specifically, Gs adjacent to 5' side of a purine, especially 5'-GG-3', were more reactive than those with 3' pyrimidine neighbors. This 5'-GG-3' effect has been observed in radical, metal-mediated, photochemical (for example, Figure 2), and ionizing radiation reactions, but only holds true for duplex DNA as the substrate. This phenomenon has been explained by our group as being due to the π-stacking interaction of the two guanine bases. This implies that 5'-G of 5'-GG-3' is a sink in hole migration through DNA, i.e. an electron-loss center created in B-form DNA would end up predominantly on 5'-G of GG steps. It is very important that the HOMO of stacked -GG- or -GOG- is always localized predominantly on the 5'-side G, which is compatible with the observed 5'-G specific DNA cleavage. The ionization potentials calculated for stacked base pairs are listed in Table 1, and for comparison, oxidation potentials, calculated based on the ionization potential data, were also shown.

Table 1. Calculated Ionization and Oxidation Potentials for Stacked Nucleobases

<table>
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<tr>
<th>Sequence</th>
<th>I.P. (eV)</th>
<th>E(O) (V vs NHE)</th>
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<tr>
<td>GGGG</td>
<td>6.98</td>
<td>0.57</td>
</tr>
<tr>
<td>GGG</td>
<td>7.07</td>
<td>0.64</td>
</tr>
<tr>
<td>GG</td>
<td>7.28</td>
<td>0.82</td>
</tr>
<tr>
<td>GA</td>
<td>7.51</td>
<td>1.00</td>
</tr>
<tr>
<td>GC</td>
<td>7.68</td>
<td>1.15</td>
</tr>
<tr>
<td>GT</td>
<td>7.69</td>
<td>1.16</td>
</tr>
<tr>
<td>G</td>
<td>7.75</td>
<td>1.20</td>
</tr>
</tbody>
</table>

⑨ Sequences are 5' to 3' in stacked B-helix geometry. ⑩ From ref 23. Calculated from data in previous column using E(O) = 0.827 x I.P. - 5.20 (adjusted for V vs NHE). ⑥

The susceptibility of each G of 5'->G,G,G-3' sequence to one-electron oxidation increased in the order G₂ > G₁ >> G₃ with a G₂:G₃ ratio of 58:42. This is not complete.
agreement with the calculated HOMO. However, the ab initio calculations indicated that the most stable cation radical is G\textsuperscript{2+} cation radical owing to the stacking stabilization by the two G bases\textsuperscript{19}. This prediction, referred to as ‘guanine-guanine (G\textsuperscript{2+}G) stacking rule’,\textsuperscript{19} has been borne out in various experiments\textsuperscript{15, 19, 22}. In the case studied, guanine radical cation is the implied intermediate that is initially formed upon oxidation. It is not clear if the same trend will be observed with oxidants that operate by other than one-electron transfer, e.g., o xo transfer. The common pattern of these 5\textsuperscript{'}-GG-3\textsuperscript{'} specific DNA cleavages strongly suggests that such specificity is not determined by the binding orientation of the DNA cleavage agents but must originate from a common intrinsic chemical property of DNA itself, which has not been well-recognized.

Unlike proteins, DNA has unique characteristics that have invited much speculation about its conducting abilities. Its double-helix ladder connected by base-pair rungs creates a neat stack of \pi electrons associated with the base pairs, prompting chemists to wonder if DNA could possibly serve as an electrical conduit through which electrons could zip unimpeded over long distances—in essence, a molecular wire. Over the past few years, the issue of DNA’s conductivity has generated heated debate among chemists as provocative evidence both for and against the wire hypothesis is now piling in from labs around the world\textsuperscript{26}. Barton et al.,\textsuperscript{21, 27} as well as Schuster et al.,\textsuperscript{28} have observed that oxidation damage can occur at G bases that are far away from the oxidant. They showed long range electron transfer has been observed by covalently tethering an oxidant to one end of an oligodeoxynucleotide duplex and observed guanine oxidation at GGG and GG sites that are up to 11 nucleotides distant. Such a far reaching translocation of charge can not be brought about by superexchange,\textsuperscript{29} the mechanism which is considered to be responsible for the strong distance dependence of the hole transfer rates to G bases in the experiments of Lewis and Wasielewski et al.,\textsuperscript{20} Tanaka et al.,\textsuperscript{31} and Giese et al.\textsuperscript{32} Recently, Giese et al. examined Norrish I photocleavage of a 4\textsuperscript{'-acylated nucleobases which is incorporated into the double-stranded DNA and proposed that in biological relevant systems, where guanine radical cation is the oxidant, long-range oxidation can occur in DNA double strands via a hopping process between the G bases and the number of AT pairs that separate the individual G bases governs the rate of each charge-transfer step according to the superexchange mechanism and that the overall charge transport is a multistep hopping process between the G bases where the individual steps contribute to the overall rate and the efficiency of this overall charge transfer does not exhibit the strong exponential dependence on the distance which is characteristic for the individual charge-transfer steps.\textsuperscript{33}

After that, many papers have been published by Barton et al.,\textsuperscript{34} Schuster et al.,\textsuperscript{35} Lewis and Wasielewski et al., Michel-Beyerle and Giese et al.,\textsuperscript{7} and our group.\textsuperscript{18} But this theme, “Is DNA insulator or wire?”, requires additional research before finding a final conclusion.

It is very important to know the chemical properties of DNA itself, such as electronic conductivity and influence upon oxidative damage. For example, in long-range hole (radical cation) migration studies as mentioned above, GG or GGG sequences are used as a hole trap because these sequences are low oxidation potential sites in duplex DNA.\textsuperscript{31} In spite of a large number of the studies on the DNA oxidizing agents and DNA alkylating agents, there are only a few studies on the properties of DNA itself. Therefore, ab initio calculation and oxidation experiments of DNA oligomer were carried out in order to investigate the property on the oxidative damage in B-form DNA duplex.

This thesis consists of five chapters on the studies on the one-electron oxidation of DNA and DNA alkylation.

In chapter 1, mapping of the hot spots for DNA damage by one-electron oxidation: efficacy of GG doublets and GGG triplets as a trap in long-range hole migration is reported. Photoinduced one-electron oxidation of various G- and GG-containing segments (5\textsuperscript{'-TXGYT-3\textsuperscript{'}}) using riboflavin as an electron-accepting photosensitizer was carried out. Relative susceptibility of each 5\textsuperscript{'-TXGYT-3\textsuperscript{'} sequence to photoinduced one-electron oxidation was in good agreement with the calculated ionization potentials.

In chapter 2, experimental and theoretical studies on the selectivity of GGG triplets toward one-electron oxidation in B-form DNA is discussed. The selectivity of 5\textsuperscript{'-TGGGT-3\textsuperscript{' and 5\textsuperscript{'-CGGGGC-3\textsuperscript{'} sequences toward photoinduced one-electron oxidation was examined experimentally and by ab initio molecular orbital calculations. It was shown that the drastic difference of the electron population in the radical orbitals of 5\textsuperscript{'-CGGGC-3\textsuperscript{'}
caused by the stacking interaction with the 5'-side G of the opposite strand can explain why G₁ is more reactive than G₂ in 5'-CG₁G₂G₃C-3' sequences.

In chapter 3, mapping of highest occupied molecular orbitals of duplex DNA by cobalt-mediated guanine oxidation was described. Ab initio calculations of HOMOs of the G-containing sequences with B-form geometry were performed and the DNA cleavage data obtained from the oxidation of duplex ODNs with Co(II) ion in the presence of benzoyl peroxide (BPO) correlated nicely with calculated HOMOs.

In chapter 4, mapping of the hot spots for DNA-RNA hybrid and quadruplex DNA by cobalt-benzoyl peroxide oxidation was described. Application of Co(II)-BPO method and photoinduced one-electron oxidation in B-form DNA to the studies of the DNA/RNA hybrid and quadruplex DNA was carried out. In DNA-RNA hybrid, the DNA cleavage pattern had a very similar tendency to the calculated HOMOs of the G-containing sequences. In quadruplex DNA, only 5'-G of GGG or GGGG was selectively cleaved, which was found for the first time.

In chapter 5, guanine selective DNA alkylation by naphthalimide and naphthaldimide derivatives possessing epoxy side chain was discussed. The naphthalimide and naphthaldimide derivatives possessing enantiomeric epoxy side chain were synthesized. The sequence selectivity of guanine alkylation by these compounds was analyzed by means of PAGE. All guanines in DNA were cleaved by using both enantiomeric epoxy naphthalimide derivatives which can not intercalate. On the other hand, 5'-G or 3'-G selective cleavage in 5'-GG-3' sequence was observed with enantiomeric epoxy naphthaldimide derivative which act as an intercalator.

References


Chapter 1

Mapping of the Hot Spots for DNA Damage by One-Electron Oxidation: Efficacy of GG Doublets and GGG Triplets as a Trap in Long-Range Hole Migration

Abstract: It is important to know accurately the relative efficiency of various GG-containing sequences that act as a trap in long-range hole migration through the DNA helix caused by one-electron oxidation. To execute precise mapping of such G-rich "hot spots", I examined the photoinduced one-electron oxidation of various G- and GG-containing DNA segments (5'-TXGYT-3') using riboflavin as an electron-accepting photosensitizer. Quantitative densitometric assay of the DNA cleavage bands revealed the relative susceptibility of each 5'-TXGYT-3' sequence to photoinduced one-electron oxidation. I also performed ab initio calculations of base paired 5'-TXGYT-3' 5-mers at the HF/6-31G* level. A linear correlation between the relative susceptibility to one-electron oxidation versus the calculated ionization potentials has been obtained. The experimental and calculated data described in Table 1 and 2 provide a simple way to predict how different sequences of 5'-XGY-3' alter the ionization potentials and the relative reactivity toward one-electron oxidation. These data are useful in estimating the relative hole-trapping efficiency of other G-containing sequences in long-range DNA oxidation by oxidizing agents, ionizing radiation and high intensity laser irradiation.
Introduction

There has been much current interest in the long-range oxidative damage to DNA through the DNA duplex caused by one-electron oxidations.1 Hole (radical cation) migration through the DNA duplex has been suggested to play a crucial role in mutagenesis and carcinogenesis caused by carcinogenic agents, ionizing radiation, and high intensity laser irradiation.2,3 As is well known, guanine (G) is the most easily oxidized base,1 and the electron loss center created in duplex DNA ultimately ends up at G residues via hole migration through the DNA duplex. Several years ago, our laboratory demonstrated both experimentally and by ab initio calculations that 5'-G residues of 5'-GG-3' steps in B form DNA are the most easily oxidized due to the GG stacks and can act as thermodynamic sinks in hole migration across the DNA π stack.4 We also demonstrated the highest occupied molecular orbital (HOMO) of a GG stack is especially high in energy and concentrated on the 5' G.4

Thereafter, examples of 5'-G selective oxidations have been reported in many different systems. These include i) photooxidation using different types of DNA-binding agents such as Rh (III)-metallointercalators,1ab-1f substituted anthraquinones,1a-5 riboflavin,6 naphthalimide derivatives,4 a p-cyano substituted benzophenone,3 and pterins,8 ii) chemical oxidation by Ru (III)-metallointercalators,1c-9 and Ni (II)-ligand/sulfite system,10 iii) two photon photoionization of DNA with a high intensity laser pulse (266 nm),11 and iv) direct irradiation with a powerful 193 nm excimer laser.12 Notably, the 5'-G selectivity of 5'-GG-3' steps is irrelevant to the structural features of the photosensitizers or the oxidizing agents, as is most typically exemplified by the two photon photoionization of duplex DNA without any additive.13

Base radical cation, formed initially by one-electron oxidation, "hops" through the duplex DNA until it is localized at a GG step (low oxidation potential site) where the radical cation reacts irreversibly with molecular oxygen or water. It is therefore very important to know accurately the relative efficiency of various G- and GG-containing sequences that act as a trap in long-range hole migration through the DNA helix, since oxidation potentials of GG steps are strongly influenced by adjacent 5'- and 5'-base pairs. To execute precise mapping of such G-rich hot spots, I have examined the photoinduced one-electron oxidation of various G- and GG-containing DNA segments.

Results and Discussion

Experimental Mapping of the Hot Spots for DNA Damage by One-Electron Oxidation Using Riboflavin

Photoinduced DNA cleavage of double-stranded 32P-end-labeled ODN 30-mers possessing two different G-containing sequences (5'-TXGYT-3') and a 5'-TTGGT-3' step as a standard (kref = 1.0) on the same strand has been carried out using riboflavin as an electron-accepting photosensitizer. The results were shown in Figure 1–7. Under the low conversion photoirradiation conditions only the cleavage bands of 5'-Gs of the two GG steps and of the middle G of the GGG triplet were observed by hot piperidine treatment. Quantitative densitometric assay of the DNA cleavage bands in several lanes (lanes 2–8) provided the average values of the relative reactivity of each G-containing sequence. In order to obtain more accurate data, the positions of these two 5'-TXGYT-3' steps were exchanged with each other but the position of standard 5'-TTGGT-3' remained unchanged on an alternative 32P-end labeled ODN 30-mer, which was similarly photooxidized with riboflavin after annealing with its complementary strand. The average values of the relative efficiency of DNA cleavage in both runs were calculated, and the entire data set of the 5'-TXGYT-3' sequences are listed in Table 1. It is clear from Table 1 that the susceptibility of the G-containing sequences to photoinduced one-electron oxidation increases in the following order: GGG > CGG > AGG > TGG > GGT > GGA > GGC > CGA > AGA > TGA > AGT > AGC.13 Pyrimidine-G-pyrimidine sequences such as TGT, TGC, CGT and CGC are almost unreactive under the photolysis
Similar DNA cleavage data have been obtained when a naphthalimide derivative\(^6\) or a p-cyano-substituted benzophenone\(^7\) was used as a photosensitizer (Figure 8), suggesting that the cleavage selectivity and reactivity are irrelevant to the binding orientations of these photosensitizers to DNA.

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**Figure 1.** Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the \(^{32}\)P-5'-CGACTCGTTGCTGTCTTTCTAT-3' sequence after photooxidation of the duplex in the presence of riboflavin. \(^{32}\)P-5'-end-labeled ODN 30-mer was hybridized to the complementary strand (5.0 \(\mu\)M, strand concentration) in an aerated buffer of 10 mM sodium cacodylate, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The \(^{32}\)P-5'-end-labeled ODN duplex (2.0 \(\times\) \(10^5\) cpm) containing riboflavin (50 \(\mu\)M) and calf thymus DNA (10 \(\mu\)M, base concentration) was irradiated at 366 nm with a transilluminator at 0 °C for 20 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lane 1) Maxam–Gilbert sequencing reactions G+A; (lanes 2–8) irradiated DNA in the presence of riboflavin; (lane 9) irradiated DNA with no riboflavin, irradiated at 366 nm for 20 min; (lane 10) DNA, dark control, no piperidine treatment. Reactivity of 5'TXGYT 3'sites was obtained from the average values of lanes 2–8.

**Figure 2.** Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the \(^{32}\)P-5'-CGACTCGTTGCTGTCTTTCTAT-3' sequence after photooxidation of the duplex in the presence of riboflavin. \(^{32}\)P-5'-end-labeled ODN 30-mer was hybridized to the complementary strand (5.0 \(\mu\)M, strand concentration) in an aerated buffer of 10 mM sodium cacodylate, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The \(^{32}\)P-5'-end-labeled ODN duplex (2.0 \(\times\) \(10^5\) cpm) containing riboflavin (50 \(\mu\)M) and calf thymus DNA (10 \(\mu\)M, base concentration) was irradiated at 366 nm with a transilluminator at 0 °C for 20 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lane 1) Maxam–Gilbert sequencing reactions G+A; (lanes 2–8) irradiated DNA in the presence of riboflavin; (lane 9) irradiated DNA with no riboflavin, irradiated at 366 nm for 20 min; (lane 10) DNA, dark control, no piperidine treatment. Reactivity of 5'TXGYT 3'sites was obtained from the average values of lanes 2–8.
Figure 3. Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the \(32^P\)-\(5'\)-CGTACTCTTTGGATGGTCTTTCTAT-\(3'\) sequence after photooxidation of the duplex in the presence of riboflavin. \(32^P\)-\(5'\)-end-labeled ODN 30-mer was hybridized to the complementary strand (5.0 \(\mu\)M, strand concentration) in an aerated buffer of 10 mM sodium cacodylate, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The \(32^P\)-\(5'\)-end-labeled ODN duplex (2.0 \(\times\) \(10^5\) cpm ) containing riboflavin (50 \(\mu\)M) and calf thymus DNA (10 \(\mu\)M, base concentration) was irradiated at 366 nm with a transilluminator at 0 °C for 20 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lane 1) Maxam-Gilbert sequencing reactions G+A; (lanes 2-5) irradiated DNA in the presence of riboflavin; (lane 6) irradiated DNA with no riboflavin, irradiated at 366 nm for 20 min; (lane 7) DNA, dark control, no piperidine treatment. Reactivity of \(5'\)TXGYT\(3'\) sites was obtained from the average values of lanes 2-5.

Figure 4. Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the \(32^P\)-\(5'\)-CGTACTCTTTGGATGGTCTTTCTAT-\(3'\) sequence after photooxidation of the duplex in the presence of riboflavin. \(32^P\)-\(5'\)-end-labeled ODN 30-mer was hybridized to the complementary strand (5.0 \(\mu\)M, strand concentration) in an aerated buffer of 10 mM sodium cacodylate, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The \(32^P\)-\(5'\)-end-labeled ODN duplex (2.0 \(\times\) \(10^5\) cpm ) containing riboflavin (50 \(\mu\)M) and calf thymus DNA (10 \(\mu\)M, base concentration) was irradiated at 366 nm with a transilluminator at 0 °C for 20 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lane 1) Maxam-Gilbert sequencing reactions G+A; (lanes 2-5) irradiated DNA in the presence of riboflavin; (lane 6) irradiated DNA with no riboflavin, irradiated at 366 nm for 20 min; (lane 7) DNA, dark control, no piperidine treatment. Reactivity of \(5'\)TXGYT\(3'\) sites was obtained from the average values of lanes 2-5.
Figure 5. Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the 32P-5'-CGTACTCTTTGCTGATAGTGGTTCTTCTAT-3' sequence after photooxidation of the duplex in the presence of riboflavin. 32P-5'-end-labeled ODN 30-mer was hybridized to the complementary strand (5.0 μM, strand concentration) in an aerated buffer of 10 mM sodium cacodylate, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing riboflavin (50 μM) and calf thymus DNA (10 μM, base concentration) was irradiated at 366 nm with a transilluminator at 0 °C for 20 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lane 1) Maxam-Gilbert sequencing reactions G+A; (lanes 2-5) irradiated DNA in the presence of riboflavin; (lane 6) irradiated DNA with no riboflavin, irradiated at 366 nm for 20 min; (lane 7) DNA, dark control, no piperidine treatment. Reactivity of 5 T'XGYT 3' sites was obtained from the average values of lanes 2-5.

Figure 6. Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the 32P-5'-CGTACTCTTTGCTGATAGTGGTTCTTCTAT-3' (left) and 32P-5'-CGTACTCTTTGCTGATAGTGGTTCTTCTAT-3' (right) sequences after photooxidation of the duplex in the presence of riboflavin. 32P-5'-end-labeled ODN 30-mer was hybridized to the complementary strand (5.0 μM, strand concentration) in an aerated buffer of 10 mM sodium cacodylate, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing riboflavin (50 μM) and calf thymus DNA (10 μM, base concentration) was irradiated at 366 nm with a transilluminator at 0 °C for 20 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lanes 1, 14) DNA, dark control, no piperidine treatment; (lanes 2, 13) irradiated DNA with no riboflavin, irradiated at 366 nm for 20 min; (lanes 3-6, 9-12) irradiated DNA in the presence of riboflavin; (lanes 7, 8) Maxam-Gilbert sequencing reactions G+A. Reactivity of 5 T'XGYT 3' sites was obtained from the average values of lanes 3-6 and 9-12.
Figure 7. Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the $^{32}$P-5'-CGTACTCTTATGTTTCTTTCTAT-3' (left) and $^{32}$P-5'-CGTACTCTTATGTTTCTTTCTAT-3' (right) sequence after photooxidation of the duplex in the presence of riboflavin. $^{32}$P-5'-end-labeled ODN 30-mer was hybridized to the complementary strand (5.0 μM, strand concentration) in an aerated buffer of 10 mM sodium cacodylate, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The $^{32}$P-5'-end-labeled ODN duplex ($2.0 \times 10^5$ cpm) containing riboflavin (50 μM) and calf thymus DNA (10 μM, base concentration) was irradiated at 366 nm with a transilluminator at 0 °C for 20 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lanes 1, 14) DNA, dark control, no piperidine treatment; (lanes 2, 13) irradiated DNA with no riboflavin, irradiated at 366 nm for 20 min; (lanes 3-6, 9-12) irradiated DNA in the presence of riboflavin; (lanes 7, 8) Maxam–Gilbert sequencing reactions G+A. Reactivity of $^{3}$TXGYT$^{3'}$ sites was obtained from the average values of lanes 3-6 and 9-12.

Figure 8. Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the $^{32}$P-5'-CGTGCTTCATTGGGTTGGTTGA TT AGTTCGTTGTTT ACTCT-3' sequence after photooxidation of the duplex in the presence of photosensitizers. $^{32}$P-5'-end-labeled ODN 41-mer was hybridized to the complementary strand (5.0 μM, strand concentration) in an aerated buffer of 10 mM sodium cacodylate, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The $^{32}$P-5'-end-labeled ODN duplex ($2.0 \times 10^5$ cpm) containing photosensitizers and calf thymus DNA (10 μM, base concentration) was irradiated at 366 nm or 312 nm with a transilluminator at 0 °C. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (lane 1) Maxam–Gilbert sequencing reactions G+A; (lane 2) irradiated DNA in the presence of 1 (50 μM, 366 nm, 20 min); (lane 3) irradiated DNA in the presence of 2 (25 μM, 312 nm, 5 min); (lane 4) irradiated DNA in the presence of 3 (100 μM, 366 nm, 30 min); (lane 5) irradiated DNA without photosensitizer (312 nm, 10 min); (lane 6) irradiated DNA without photosensitizer (366 nm, 30 min); (lane 7) DNA, dark control, no piperidine treatment.
Table 1: Relative Reactivity of 5'-TXGYT-3' Sequences in B
Form DNA toward Photoinduced One-Electron Oxidation

<table>
<thead>
<tr>
<th>X (5'-side)</th>
<th>Y (3'-side)</th>
<th>G</th>
<th>A</th>
<th>T</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>TGGGT</td>
<td>2.7 ± 0.1</td>
<td>0.80 ± 0.02</td>
<td>0.90 ± 0.03</td>
<td>0.70 ± 0.03</td>
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<tr>
<td>A</td>
<td>TAGGT</td>
<td>1.00 ± 0.06</td>
<td>0.4 ± 0.2</td>
<td>0.20 ± 0.04</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>T</td>
<td>TGGGT</td>
<td>1.00 ± 0.07</td>
<td>0.30 ± 0.07</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>C</td>
<td>TCGGT</td>
<td>2.0 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a 5'-32P-end labeled ODN 30 mers containing two 5'-TXGYT-3' segments and a 5'-TTGGGT-3' site as a standard (krel=1.0) were prepared. Riboflavin-sensitized photooxidation of these oligomers after annealing with complementary strands was conducted as described in Figure 1-7. The relative reactivity was estimated from each 5'-TXGYT-3' segment under the low conversion photoirradiation conditions. n.d. = no data available.

Ab Initio Calculation of 5'-TXGYT-3' Sequence

I next examined the ab initio calculations of sixteen sets of base paired G- and GG-containing 5-mers at the HF/6-31G* level using GAUSSIAN94. While calculated HOMO energies of monomeric DNA nucleobases have been reported to correlate well the experimental vertical IPs, only a few ab initio calculations on stacked nucleobases have been reported. Double strands of B-form DNA with the 5'-TXGYT-3' sequence were built using Insight II program with standard B-form parameters. For quantum mechanical calculations, all the sugar backbones of the duplex 5-mer were removed from the coordinate file, keeping the positions of all atoms fixed, and were replaced by methyl groups. Calculated ionization potentials (IPs) estimated by Koopmans' theorem are shown in Table 2. The calculated IPs are in the following order: GGG (6.34 eV) < CGG (6.44 eV) < AGG = GGA (6.50 eV) < TGG (6.52 eV) < GTG (6.59 eV) < AGC = CGA (6.63 eV) < AGA (6.73 eV) < TGA (6.76 eV) < CGT (6.91 eV) < AGT (6.93 eV) < CGC = TGT (6.96 eV) < AGC (6.97 eV) < TGC (7.12 eV).

Table 2: Calculated Ionization Potentials (IPs) of Stacked Base Paired
Deoxypentanucleotides (5'-TXGYT-3') (eV)

<table>
<thead>
<tr>
<th>X (5'-side)</th>
<th>Y (3'-side)</th>
<th>G</th>
<th>A</th>
<th>T</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>TGGGT</td>
<td>6.34</td>
<td>6.50</td>
<td>6.59</td>
<td>6.63</td>
</tr>
<tr>
<td>A</td>
<td>TAGGT</td>
<td>6.50</td>
<td>6.73</td>
<td>6.93</td>
<td>7.01</td>
</tr>
<tr>
<td>T</td>
<td>TGGGT</td>
<td>6.52</td>
<td>6.76</td>
<td>6.96</td>
<td>7.12</td>
</tr>
<tr>
<td>C</td>
<td>TCGGT</td>
<td>6.44</td>
<td>6.63</td>
<td>6.91</td>
<td>6.96</td>
</tr>
</tbody>
</table>

a Ionization potentials were estimated by Koopmans' theorem. The values are the HOMO energies of HF/6-31G* single-point calculations. IPs of the corresponding 5-mer sequences (e.g. TGGGT) could not be obtained accurately since the complementary strand (e.g. AACGA) has a lower IP. Therefore, only three base pairs (e.g. TGC / AGC) were calculated in these cases.

Since the hole is trapped at lower oxidation potential sites on the DNA helix, hole-trapping efficiency in long-range DNA oxidation would follow the same order, i.e., GGG and CGG are deep hole traps (low ionization potential sites) and AGG is a better hole trap than GGC. The calculated IPs are in fairly good agreement with the experimentally obtained relative reactivity. A plot of the experimentally obtained relative reactivity (krel) versus calculated IPs is illustrated in Figure 9. A linear correlation between the log of the relative rate constants of one-electron oxidation and the calculated IPs has been obtained.
Conclusion

The present experimental and calculated data provide a simple way to predict how different sequences of 5'-XGY-3' alter the IPs and, as a result, the reactivity toward one-electron oxidation. These data are very useful in predicting the favorable sites for one-electron oxidation of DNA and in estimating the relative hole-trapping efficiency of a number of other G-containing sequences in long-range DNA oxidation when the hole is created by oxidizing agents, ionizing radiation, and high-intensity laser irradiation, although the DNA cleavage efficiencies do not necessarily correlate to the hole-trapping efficiencies. The present results shed light on the quantitative aspects of DNA oxidation and the effectiveness of GG-doublets and GGG-triplets as a trap in long-range hole migration.

Experimental Section

General: Riboflavin was purchased from Nacalai Tesque Co., Ltd. Calf thymus DNA (1 g) was purchased from Pharmacia Biotech. Cyano-substituted benzophenone\(^1\) and naphthalimide derivative\(^2\) were synthesized as reported previously. The oligodeoxynucleotides were purchased from Greiner Japan Co. Ltd. T4 polynucleotide kinase was purchased from NIPPON GENE (10 units/\(\mu\)L) and \(\gamma\)\(^{32}\)P-ATP (10 mCi/mL) was from Amersham. All aqueous solutions utilized purified water (MILLIPORE, Mili-Q sp UF). Photoirradiation at 366 nm was carried out using a Cosmo BIO CSF-20AF transilluminator. Photoirradiation at 312 nm was carried out using a Vilber Lourmat TFX-20M transilluminator. A GIBCO BRL Model S2 sequencing gel electrophoresis apparatus was used for polyacrylamide gel electrophoresis (PAGE). The gels were analyzed by autoradiography with a Bio-Rad Model GS-700 imaging densitometer and Bio-Rad Molecular Analyst software (Version 2.1).

Theoretical Calculations

Ab initio calculations were performed at the HF/6-31G* level for other calculations utilizing the GAUSSIAN 94 program package.\(^{14}\) Geometries of the duplex consisting of stacked methylated nucleobases at N\(_1\) (pyrimidine base) and N\(_8\) (purine base) were constructed as follows: the corresponding duplex 5-mers were built using the insight II program (Version 97.0) with standard B-form helical parameters (pitch, 3.38 Å; twist, 36°; tilt, 1°) which have been optimized by X-ray crystallographic analysis of relevant monomers and X-ray diffraction data of polymers.\(^{17}\) All the sugar backbones of the duplex were removed except for the deoxyribose C1' carbon and C1' H and two H atoms were then attached to the C1' methine to complete stacked N-methylated nucleobases, keeping the position of all atoms fixed. HOMOs of the calculated 5-mers were displayed graphically using Gaussian11F.
Preparation of 5'-32P-End-Labeled ODN

The oligodeoxynucleotides (ODNs, 400 pmol strand concentration) were 5'-end-labeled by phosphorylation with 4 μL of [γ-32P]ATP and 4 μL of T4 polynucleotide kinase using standard procedures. The 5'-end-labeled ODNs were recovered by ethanol precipitation and further purified by 15% preparative nondenaturing gel electrophoresis and isolated by the crush and soak method.

Cleavage of 32P-5'-End-Labeled ODNs by Photoirradiation in the Presence of Photosensitizers

32P-5' end-labeled ODNs were hybridized to the complementary strand (2.5 μM, strand concentration) in 10 mM sodium cacodylate buffer, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The 32P-5'-end-labeled ODN duplexes (2.0 × 10^5 cpm) containing a photosensitizer and calf thymus DNA (10 μM, base concentration) (total volume 120 μL) was irradiated with a transilluminator at 0 °C. After irradiation, all reaction mixtures were ethanol precipitated with the addition of 10 μL of 3 M sodium acetate and 800 μL of ethanol. The precipitated DNA was washed with 100 μL of 80% cold ethanol and dried in vacuo. The precipitated DNA was resuspended in 50 μL of water or 10% piperidine (v/v), heated at 90 °C for 20 min, evaporated by vacuum rotary evaporation to dryness. The radioactivity of the samples was then assayed using an Aloka 1000 liquid scintillation counter and the dried DNA pellets were resuspended in 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). All reactions, along with Maxam-Gilbert G+A sequencing reactions, were heat denatured at 90 °C for 3 min and quickly chilled on ice. The samples (1–2 μL, 2.5 × 10^3 cpm) were loaded onto 12% polyacrylamide/7 M urea sequencing gels and electrophoresed at 1900 V for approximately 2 h and transferred to a cassette and stored at −80 °C with Fuji X-ray film (RX-U). The gels were analyzed by autoradiography with a densitometer and BIORAD Molecular Analyst software (version 2.1). The intensities of the spots resulting from piperidine treatment were determined by volume integration.
References and Notes


(13) The data for the segments containing four contiguous Gs, i.e., 5’-GGGG-3’, are not listed in the Table since such G-rich segments may exist locally in a different conformation such as in A-form DNA.


Chapter 2

Experimental and Theoretical Studies on the Selectivity
of GGG Triplets toward One-Electron Oxidation in B-Form DNA

Abstract: Selectivity of 5'-TGGGT-3' and 5'-CGGGC-3' sequences toward photoinduced one-electron oxidation was examined experimentally and by ab initio molecular orbital calculations. It was confirmed experimentally that G_2 of 5'-TG_1G_2G_3T-3' is more reactive than G_1, while for 5'-CG_1G_2G_3C-3' the ordering of the selectivity is reversed, that is, G_1 > G_2. The ab initio MO analyses were performed to elucidate the difference of the selectivities between 5'-TGGGT-3' and 5'-CGGGC-3' sequences. For 5'-TGGGT-3' sequence, the spin densities of G_1 and G_2 in neutral radical (5'-TG_1G_2G_3-3') have a similar pattern and the shapes of the corresponding radical orbitals are also very similar. It was concluded that the selectivity is due to the stability of the (5'-TG_1G_2G_3-3') neutral radicals, that is, the 5'-TG_1G_2G_3-3' is more stable in energy than the 5'-TG_1G_2G_3-3'. For 5'-CGGGC-3' sequence, it was found that the spin density on N_1 of G_1 in neutral radical (5'-CG_1G_2G_3-3') is distinguishably different from the corresponding spin density of G_2, which has a similar pattern to those of G_1 in 5'-TG_1G_2G_3-3'. The radical orbital (SOMO) of G_1 is delocalized on guanine base and up to the paired cytosine base, while the radical orbital of G_2 is essentially localized on guanine base. This drastic difference of the electron population in the radical orbitals caused by the stacking interaction with 5'-side G of the opposite strand can explain why G_1 is more reactive than G_2 in 5'-CG_1G_2G_3-3' sequence.
Introduction

Long-range DNA damage caused by one-electron oxidation of nucleobases has been extensively studied in the viewpoint of mutagenesis and carcinogenesis induced by carcinogenic agents, ionizing radiation, photosensitization with endogenous photosensitizers, and high-intensity laser irradiation. Since guanine is the most easily oxidized base among DNA nucleobases, guanine radical cation is the initial product of DNA one-electron oxidation in a wide variety of systems. The electron-loss center created in DNA duplex by one-electron oxidation ultimately moves to end up at guanine (G) base via hole migration through the DNA π stack. As is well known, 5'-G of 5'-GG-3' sequences is selectively oxidized in the B-form DNA in the reaction systems using a variety of oxidizing agents. Precise mapping of such GG-containing hot spots in one-electron oxidation of B-form DNA has also been accomplished. These GG doublets are often used as a probe for the terminus in the long-range hole migration. It was also demonstrated that 5'-GGG-3' triplets act as a more effective trap in hole migration than 5'-GG-3' doublets. For example, Kawanishi and coworkers have observed that the central Gs of 5'-AGGG-3', 5'-AGGGT-3', and 5'-TGGGT-3' sequences are selectively damaged under the photosensitization with riboflavin and pterin. Spassky and coworkers have also shown that the direct two-photon excitation of B-form DNA with high-energy laser pulse leads to the damage of central G in a variety of 5'-GGG-3' triplets.

On the other hand, it was reported by Barton and coworkers that the duplexes containing 5'-CGGGC-3' sequence are primarily cleaved at 5'-side G using Rh and Ru metallo-intercalators. This is conflicted with our previous results that the central G is selectively damaged in 5'-CGGGC-3' sequence are primarily cleaved at 5'-side G using Rh and Ru metallo-intercalators. A linear correlation between the relative susceptibility toward one-electron oxidation of GGG triplets and GGG-triplets act as an effective trap in long-range hole migration in DNA one-electron oxidation.

Theoretical calculations of DNA bases have been extensive for ionization potentials of monomer nucleobases, stability of nucleobase-pair in neutral and radical cation states, and for stacking interactions between nucleobases. However, only a few ab initio calculations on stacked nucleobases have been reported. Theoretical analyses for one-electron oxidation of GGG triplets are scarcely found.

In this paper, we examined the detail analyses of the selectivities of 5'-TG, 5'-TGGGT-3' and 5'-CG, 5'-CGGC-3' sequences toward photoinduced one-electron oxidation. It was confirmed experimentally that G, of 5'-TG, 5'-TGGGT-3' is more reactive than G, while for 5'-CG, 5'-CGGC-3' the ordering of the selectivity is reversed, that is, G, > G, in consistent with previous results. In both sequences, the 3'-side G (G,) was far less reactive than G, and G, respectively. We next performed the ab initio MO analyses to elucidate the reason why the selectivity of 5'-TG, 5'-CGGC-3' is different from that of 5'-TGGGT-3'.

Results and Discussion

Experimental Results

5'-32P-end-labeled ODN 33-mer which includes simultaneously two triplet sequences of 5'-TGGGT-3' and 5'-CGGGC-3' and one doublet sequence of 5'-TGG-3' was hybridized to the
complementary strand in an aerated buffer of sodium cacodylate. In the presence of photosensitizer such as riboflavin (1) and cyano substituted benzophenone (2), the 5'-32P-end-labeled ODN duplex was photoirradiated under the conditions shown in Fig. 1. After hot piperidine treatment, the mixture was analyzed by gel electrophoresis. Figure 1 (a) shows the autoradiogram, whereas Fig. 1 (b) illustrates the selectivity for the damage on two GGG triplets and GG doublet induced by photoirradiated riboflavin.

It can be easily seen from Fig. 1 that the guanine base is selectively damaged. The observed selectivities TG1G2T (G1 > G2), TG1G2G3T (G2 > G1) and CG1G2G3C (G1 > G2) are consistent with the previously reported results of GGG triplets. It was also confirmed that such selectivity is not altered by the photosensitizer used. In consistent with previously observed selectivity, 5'-CG1G2G3C-3' is an unique sequence that exhibits high G1 selectivity, in contrast to the G2 selectivity observed for other GGG triplets such as AG1G2G3T, TG1G2G3T and AG1G2G3A. In all cases, the 3'-G (G3) was far less reactive than G1 and G2. Since there is a possibility that such unique selectivity observed for 5'-CG1G2G3C-3' arises from the difference in the duplex structure such as A-form rather than normal B-form, we measured the CD spectra of the duplex oligomers, 5'-CATCGGCTTG-3'/5'-CAAGCCCGAATG-3' and 5'-ATGGGTACCCAT-3' (self complementary). As shown in Fig. 2, both duplexes gave typical CD spectra of B-form DNA, implying that the selectivity of 5'-CG1G2G3C-3' may arise from the intrinsic chemical property of this sequence.

Figure 1. (a) Autoradiograms of a denaturing gel electrophoresis for 5'-32P-end-labeled ODN 5'-CGTACTCTGGTCCGGGCTTGTTTCTTTTCTT-3' sequence after photo-oxidation of the duplex in the presence of photosensitizers, riboflavin (1) and benzophenone derivative (2). The 5'-32P-end-labeled ODN duplex containing a photosensitizer and calf thymus DNA was irradiated with a transilluminator under the conditions described in the experimental section. (Lane 1) Maxam-Gilbert sequencing reactions G+A; (lane 2) irradiated DNA in the presence of 1 (45 μM, 366 nm, 40 min); (lane 3) irradiated DNA in the presence of 2 (50 μM, 312 nm, 40 min); (lane 4) irradiated DNA without sensitizer (366 nm, 30 min); (lane 5) irradiated DNA without sensitizer (312 nm, 40 min); (lane 6) DNA, dark control, no piperidine treatment. (b) The histogram representing relative intensities of cleavage bands obtained by densitometric assay of lane 3 (benzophenone derivative sensitization).

Figure 2. CD spectra of 5'-CATCGGCTTG-3'/5'-CAAGCCCGAATG-3' (solid line) and 5'-ATGGGTACCCAT-3' (self complementary, dotted line) (150 mM, base concentration) in 10 mM sodium cacodylate buffer, NaCl 100 mM (pH 7.0) at 4°C.
Calculational Details

We performed ab initio molecular orbital calculations to elucidate the selectivities of 5'-TGGG-3' and 5'-CGGG-3' sequences toward one-electron oxidation. The geometries of 5'-TGGG-3' and 5'-CGGG-3' sequences possessing double stranded of B-form structure were constructed using Insight II program with standard B-form geometrical parameters which have been optimized by X-ray crystallographic analysis of relevant monomers and X-ray diffraction data of polymers. All the sugar back bones of the duplex 4-mer were replaced by methyl group, keeping the position of all atoms fixed. For the calculations of radical cations and deprotonated neutral radicals of the 5'-TGGG-3' and 5'-CGGG-3' sequences, the same geometries constructed by the above procedure were employed without geometry optimization. The charge densities and spin densities are summed up for every base of GGG triplet to distinguish which of the guanines has localized positive charge and spin densities corresponding to the states of the radical cation and neutral radical.

All calculations were carried out at the HF/6-31G level using Gaussian 94 program package. In our previous paper, we demonstrated that the Koopmans IPs estimated at HF/6-31G* level with polarization function are useful way to predict the relative reactivity toward one-electron oxidation of G-containing 5'-TXGYT-3'. The analysis of HOMO is also very important for GG and GGG sequences. Koopmans IP is equal to the HOMO energy with switched sign, while the vertical IP is defined by the difference in energies between the neutral ground state and the radical cation state in which the one electron is removed from the ground state. Therefore, the vertical IP depends on the calculational accuracy of the radical cation state.

In order to confirm the effects of polarization function, the Koopmans and vertical IPs of GGG-triplets were evaluated with and without the polarization functions. As can be seen from Table 1, the 6-31G* basis set gave Koopmans IPs of 6.42 and 7.06 eV for the double and single strands of GGG triplets, respectively, while the 6-31G basis set without polarization function also gave 6.58 and 7.34 eV. As the vertical IPs, the 6-31G* gave 4.98 and 5.61 eV and the 6-31G gave 5.18 and 5.88 eV for the double and single strands. Although the effects of the polarization functions are slightly larger than 0.2 eV for the single strand, they are less than 0.2 eV for the double strand. This examination shows that the 6-31G basis set is available for studying the stabilities of neutral, radical cation and neutral radical states.

Table 1. Ionization Potentials (eV) of Stacked Base Paired Oligodeoxynucleotides Calculated at the HF/6-31G Level

<table>
<thead>
<tr>
<th>base</th>
<th>Koopmans IP</th>
<th>vertical IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGGG</td>
<td>6.45</td>
<td>4.98 (TG&quot;GG)</td>
</tr>
<tr>
<td></td>
<td>5.03 (TGG&quot;G)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.44 (ACC&quot;C&quot;)</td>
<td></td>
</tr>
<tr>
<td>CGGG</td>
<td>6.39</td>
<td>5.02 (CGG&quot;G)</td>
</tr>
<tr>
<td>GGG</td>
<td>6.58 (6.42)</td>
<td>5.18 (4.98)</td>
</tr>
<tr>
<td>GGG*</td>
<td>7.34 (7.06)</td>
<td>5.88 (5.61)</td>
</tr>
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</table>

a Complementary strand of TGGG. b Estimation at HF/6-31G* level. c Single Strand.

Theoretical Analyses on Radical Cations of 5'-TGGG-3' and 5'-CGGG-3'

Table 1 summarizes the Koopmans ionization potentials (IPs) of 5'-TGGG-3' and 5'-CGGG-3' sequences. The IPs of both 5'-TGGG-3' and 5'-CGGG-3' sequences are almost same with a small difference of only 0.06 eV. The IP of 6.42 eV of double stranded GGG shown in Table 1 is smaller than 6.73 eV of (GG)/(CC) estimated previously at HF/6-31G* level, indicating that the addition of guanine base to GG-doublet considerably decreases the ionization potential, in consistent with the previous report by Houk and Foote. Addition of thymine and cytosine at the 5' side also induced an additional decrease of IP from 6.58 eV to 6.45 and 6.39 eV, respectively. These estimations mean that both sequences of TGGG and CGGG can act as thermodynamic sinks in hole migration through DNA π stack. It is, therefore, reasonable to consider that the cation radical states of 5'-TGGG-3' and 5'-CGGG-3' sequences are the first step of one-electron oxidation.
For the radical cations of XGGG (X = T and C), three possible states XG+GG, XGG+G, and XGGG+ are to be considered. The hole is expected to migrate reversibly among these radical cation states. In order to elucidate the stabilities of the radical cations of both 5'-TGGG-3' and 5'-CGGG-3' sequences, we performed the SCF calculations of three states of the radical cations. In the case of 5'-TGGG-3' sequence, the first SCF calculation is automatically converged to the state of 5'-TGG+G-3'. We tried calculations again to find the states of 5'-TG+GG-3' and 5'-TGGG+-3' by constructing the corresponding initial guesses of the SCF calculations by changing the occupation number of electrons of the molecular orbitals of 5'-TGG+-G-3' state. The expected 5'-TG+GG-3' state was obtained but 5'-TGGG+-3' was not found, as shown in Table 2. Although similar procedures were carried out for the 5'-CGGG-3' sequence, only 5'-CGG+G-3' state was found.

### Table 2

<table>
<thead>
<tr>
<th>base pairs, E_{rel}</th>
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<th>XGGG+</th>
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<td>radical cations</td>
<td>TG+GG</td>
<td>TGG+G</td>
<td>TGGG+</td>
</tr>
<tr>
<td>E_{rel}</td>
<td>0.0</td>
<td>1.3</td>
<td>not found</td>
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<tr>
<td>neutral radicals</td>
<td>TGGG</td>
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<tr>
<td>E_{rel}</td>
<td>7.8</td>
<td>0.0</td>
<td>not found</td>
</tr>
</tbody>
</table>

* Mark * means radical cation (++) or radical (•). * Total energy is -4022.32099 a.u. * Total energy is -4021.83214 a.u. * Total energy is -4038.32504 a.u. * Total energy is -4037.83535 a.u.

It can be seen from Table 2 that the 5'-TG,G,G,G,-3' state is more stable than the 5'-TG,G,G,G,-3' state but only by 1.3 kcal/mol, showing that they are considered to be isoenergetic states. The hole may be trapped by G, with equal probability in the radical cations of 5'-TGGG and the hole can reversibly move between 5'-TG,G,G,-3' and 5'-TGG,G,G,-3'. However, these results are conflict with the experimentally observed high G, selectivity for 5'-TG,G,G,G,-3'.

On the other hand, in the case of the 5'-CG,G,G,G,-3' sequence, theoretical calculations show that the hole may be trapped at only G, as indicated in Table 2. The hole, if generated at G, of largest HOMO, will rapidly pass through the radical cation state 5'-CG,G,G,G,-3' to reach 5'-CG,G,G,G,-3'. However, as a next pathway from 5'-CG,G,G,G,-3', there are at least two possibilities; (i) the hole migrates to adjacent G, to yield stable 5'-CG,G,G,G,-3' state and (ii) the deprotonation from the G, occurs to produce the corresponding neutral radical 5'-CG,G,G,G,-3'. In fact, the 5'-CG,G,G,G,-3' state is theoretically obtained as described in next section (see also Table 2). These two pathways of hole migration and deprotonation are considered to be competitive in one-electron oxidation reaction. Therefore, the theoretical calculations indicate that the experimentally observed selectivity of 5'-CGGG is expected to be caused from the stability and/or the unique electronic structure of deprotonated neutral radical state (5'-CGGG+), not due to the stability of (5'-CGGG)+ radical cation states.

It is easily seen from Table 1 that the vertical IPs of both 5'-TGGG-3' (4.98 eV) and 5'-CGGG-3' (5.02 eV) sequences are very close, being insensitive to which base is stacked at 5'-side. However, when compared with double stranded simple GGG, the thymine and cytosine at 5'-side cause the additional decrease of the vertical IPs. These effects are also found for the Koopmans IPs.

Table 3 summarizes the orbital energies of the highest occupied MO (HOMO) and the singly occupied MO (SOMO) corresponding to the radical orbitals. From Table 3, the radical orbitals of 5'-TG,G,G,G,-3', 5'-TG,G,G,-3' and 5'-CG,G,G,-3' states do not apparently coincide with their HOMOs. The radical orbitals are largely stabilized compared with the HOMOs which are corresponding to the 304-th $\alpha$ MO in both 5'-TGGG-3' and 5'-CGGG-3' sequences. For example, the radical orbitals are corresponding to the 291-th, 292-th and 291-th $\alpha$ MO in 5'-TG,G,G,-3', 5'-TG,G,G,-3' and 5'-CG,G,G,-3' states, respectively. The disagreement between
the SOMO and HOMO is not unusual. Each orbital is localized on each base in a radical cation system. The SOMO, radical orbital, which is localized on the positively charged guanine base, is largely stabilized due to the net positive charge, and apparently corresponds to the HOMO of G⁺⁺ in 5'-TG*GG-3' and 5'-TGG*G-3' and 5'-CGG*G-3' states. Therefore, the HOMO of 5'-TG*GG-3', 5'-TGG*G-3' and 5'-CGG*G-3' states comes from the HOMO of other bases except for G⁺⁺ in TGGG and CGGG sequences. This considerable energy-lowering of the radical orbitals of the radical cation G⁺⁺ is in harmony with the hopping mechanism of hole migration via guanine radical cation proposed theoretically and experimentally. The HOMOs of the 5'-TG*GG-3' and 5'-TGG*G-3' states are localized on G⁺⁺ bases of (TGGG*)/(CCCA) and (TG*GG)/(CCCA), respectively, while the 5'-CGG*G-3' state is on G⁺⁺ base of (CGGG)/(CCCG*).

Theoretical Analyses on Neutral Radicals of 5'-TGGG-3' and 5'-CGGG-3'

When the radical cation of guanine is formed in a double stranded structure, the N1 proton immediately transfers to N3 of cytosine of the base pair with pKa = 4.3. The G⁺⁺ is a strong acid with pKa = 3.9 to easily generate the neutral radical through deprotonation after one-electron oxidation in neutral pH. In fact, ESR studies have shown that the N1 proton is the one to be lost in this process. The proton is expected to be released into the solvent under our experimental condition of neutral pH.

For the 5'-TGGG-3' sequence, three neutral radical states of 5'-TG.GG-3', 5'-TGG.G-3' and 5'-TGGG.-3', which are deprotonated states from N1 of guanine, were obtained by the SCF procedures starting from the initial guess corresponding to the electronic configurations of the three radical states. It is apparent from Table 2 that 5'-TGGG-3' is the most stable among neutral radical states of 5'-TGGG-3'. The 5'-TGGG-3' is less stable by 9.1 kcal/mol than the 5'-TGGG-3', and 5'-TGGG-3' is only 2.4 kcal/mol higher than the 5'-TGGG-3'. These results for the stabilities of three radical states are in good agreement with the experimental observation that G₂ of 5'-TG₁G₂G₃-3' is the most reactive site in DNA one-electron oxidation. As discussed latter, the reactivities of 5'-TG*GG-3' and 5'-TGGG-3' toward molecular oxygen are expected to be very similar by the analyses of the radical orbitals (SOMOs) of both neutral radical states. It can be, therefore, considered that the selectivity of the 5'-TGGG-3' sequence is primarily determined by the stability of neutral radical states produced through the radical cations by one-electron oxidation.

### Table 3. Orbital Energies (a.u.) Corresponding to the Radical Orbital (SOMO) and HOMO.

<table>
<thead>
<tr>
<th>Radical Orbital</th>
<th>SOMO Energy</th>
<th>HOMO Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGGG</td>
<td>-0.23475</td>
<td>-0.23475</td>
</tr>
<tr>
<td>TGG⁺⁺G</td>
<td>-0.44725</td>
<td>-0.32288</td>
</tr>
<tr>
<td>TGG⁺⁺</td>
<td>-0.29256</td>
<td>-0.24870</td>
</tr>
<tr>
<td>CGGG</td>
<td>-0.30267</td>
<td>-0.24325</td>
</tr>
<tr>
<td>CGG⁺⁺G</td>
<td>-0.44725</td>
<td>-0.32288</td>
</tr>
<tr>
<td>CGG⁺⁺</td>
<td>-0.30267</td>
<td>-0.24325</td>
</tr>
</tbody>
</table>

From Table 3, the HOMOs of the 5'-TGGG-3' radical states are not apparently corresponding to the radical orbitals (SOMOs). The HOMOs and SOMOs are destabilized by the deprotonation, and the orbital energies of SOMOs are closer to those of HOMOs than the cases of radical cations. The HOMO of the 5'-TGGG-3' state is localized on G⁺⁺ of (TGGG*)/(CCCA), while those of the 5'-TGGG-3' and 5'-TGGG-3' are on G⁺⁺ of (TG*GG)/(CCCA).

On the other hand, although the only one state of radical cation for the 5'-CGGG-3' sequence was found, two radical states of 5'-CGG*G-3' and 5'-CGGG-3' were found as shown in Table 2. The remainder radical state of 5'-CGGG-3' was not still found. The 5'-CGGG-3' state is less stable by 7.8 kcal/mol than the 5'-CGG*G-3' state. This result is not in agreement with the
selectivity observed experimentally in DNA photo cleavage that the G₁ is more easily damaged than G₂, indicating that unlike 5'-TGGG-3' the selectivity of 5'-CGGG-3' sequence in DNA one-electron oxidation is not determined primarily by the stabilities of radical cations and neutral radicals.

It is also seen from Table 3 that the radical orbitals (SOMO) of 5'-CGGG-3' and 5'-CGGG-3' do not still coincide with the HOMOs. The SOMOs and HOMOs are highly destabilized due to the deprotonation of the corresponding radical cations. Compared with those of the radical cations, the change of orbital energies means that the radical orbitals of the neutral radical states are more reactive than those of the radical cation states. The HOMO of the 5'-CGGG-3' state is localized on G* of (CGG*)/(CCCG), while that of the 5'-CGGG-3' is on G* of (CG*(G))/(CCCG).

**Analyses of Radical Orbitals in Neutral Radical States of 5'-TGGG-3' and 5'-CGGG-3'**

The reaction mechanism of one-electron oxidation of guanine base has been proposed as shown in Scheme 1. The neutral radical dG is an important intermediate for the reaction with molecular oxygen to yield ultimately imidazolone product (dlz). Therefore, the reactivity of the neutral guanine radicals generated in the double stranded B-form DNA was investigated in more detail in order to elucidate the selectivity of 5'-TGGG-3' and 5'-CGGG-3' as well as their stabilities.

Before analyzing radical orbitals in neutral radical states, it is interesting to explore the atomic spin densities of guanine radicals in neutral radical states of the 5'-TGGG-3' and 5'-CGGG-3' sequences. Figure 3 depicts the atomic spin densities of G-radical of four neutral radical states of the 5'-TGGG-3' and 5'-CGGG-3' sequences. The C5-s of all states have relatively large spin densities from 0.667 to 0.822, in consistent with the proposed oxidation mechanism of guanine radical under aerobic conditions, i.e., molecular oxygen preferentially attacks on C5 of guanine neutral radical to yield ultimately imidazolone product (dlz) via C5 hydroperoxy intermediate (Scheme 1). The spin densities on C5-s of 5'-TGGG-3' (a) and of 5'-TGGG-3' (b) are nearly equal in each other, while those of 5'-CGGG-3' (c) and 5'-CGGG-3' (d) are different by 0.08, i.e., the spin density of C5 of 5'-CGGG-3' (c) is much larger than that of 5'-CGGG-3' (d). It can be easily seen from Fig. 3 that the patterns of spin densities are classified into two different groups. One group is a set of 5'-TGGG-3' (a), 5'-TGGG-3' (b) and 5'-CGGG-3' (d) radical states, and the other is 5'-CGGG-3' (c). The spin densities on deprotonated N1, C2 and C6 are remarkably different between these two groups. The N1 atoms in the former group have large spin densities from 0.796 to 0.829, while in the latter small value of only 0.172. C6 in 5'-CGGG-3' (c) has smaller negative spin density of -0.452 compared with ca. -0.7 for the former group. Further, C2 of 5'-CGGG-3' (c) has only half value of the corresponding atoms in the former group. However, the summations of atomic spin densities of G-radicals in both groups are nearly equal to unity, even if the spin densities on each atom are remarkably different in each other. These results apparently suggest that the electronic structures of the G-radicals are quite different between these two groups, indicating that G-radicals of both groups are expected to be located under a different interaction circumstance induced by base stacking and base pairing in the B-form DNA.
responsible for the reactivity of the oxidation reaction. Figure 4 depicts the molecular orbitals corresponding to the radical orbitals in 5'-TGGG-3' (a), 5'-TGG'G-3' (b), 5'-CG'GG-3' (c) and 5'-CGGG-3' (d) neutral radicals. It can be seen from Fig. 4 that in all cases the radical orbitals are \( \pi \)-type orbitals, which are spread over the guanine base. The radical orbital of 5'-CGG'G-3' (d) state has entirely same shape (for examples, the magnitude of robe and phase relationship) to that of 5'-TGG'G-3' (b), and slightly interacts with the imidazole ring of the guanine at 3'-side. In addition, the radical orbital of 5'-TGG'G-3' (a) is also quite similar to the above and slightly interacts with the imidazole ring of the 3'-side guanine. In fact, the phase relationships of the radical orbitals in both cases totally coincide each other. On the other hand, it is easily found from Fig. 4 that the radical orbital of the 5'-CGGG-3' (c) state is drastically different from the above three radicals, being largely delocalized on the guanine base and on the paired cytosine base as well, although it also slightly interacts with the pyrimidine ring of the 3'-side guanine. These behaviors in the radical orbitals are in consistent with those of the atomic spin densities of the guanine radical discussed above.

Figure 3. Atomic spin densities of the G'-radical in the neutral radical states of (a) 5'-TG(GG-3', (b) 5'-TGG'G-3', (c) 5'-CG'GG-3' and (d) 5'-CGG'G-3'.

Figure 4. Three dimensional views of the radical orbitals of 5'-TGGG-3' and 5'-CGGG-3' neutral radicals. Each radical orbital is viewed from the same direction that the C6-N1 bond in the G0-radical guanine is faced to the front. The green rigid circle corresponds to the carbon atom, red one is oxygen atom, and blue one is nitrogen atom. The white circle indicates hydrogen atom. (a) 5'-TG(GG-3', (b) 5'-TGG'G-3', (c) 5'-CG'GG-3' and (d) 5'-CGG'G-3'.

It is conceivable from the similarity of the radical orbitals of G1' and G2' in 5'-TG1,G2,G3'-3' neutral radical shown in Fig. 4 that the reactivity of G1' and G2' radicals toward molecular oxygen might be very similar with almost equal activation energy. It seems not unlikely that the base paired 5'-TG1,G2,G3'-3' radical would be converted reversibly to the 5'-TGG1,G2,G3'-3' radical via the radical cations (5'-TGGG-3')', although there is no direct path between the two radical states. However, under the condition of neutral pH, the neutral radical states, (5'-TGGG-3')', are
preferentially generated from the radical cations, \((5'-\text{TGGG-3'})^+\) as shown in Fig. 5. Therefore, the selectivity of the reaction in the 5'-TG,G,G,-3' sequence may be determined primarily by the difference of the stability between 5'-TG,G,G,-3' and 5'-TG,G,G,-3' states as discussed above.

Figure 5. Schematic representation of reaction pathways of 5'-TGGG-3' and 5'-CGGG-3' by one-electron oxidation.

On the other hand, as shown in Fig. 4 (c), the \(G_1\) in the 5'-CG,G,G,-3' state apparently exists in a unique interacting environment which is induced by strong stacking interaction with the 5'-side guanine of the opposite strand, unlike the \(G_1\) in the 5'-TG,G,G,-3' state. On the contrary, the \(G_1\) of 5'-CG,G,G,-3' state exists in a very similar interacting environment as \(G_1\) of 5'-TG,G,G,-3' state. This dramatic discrepancy seems to be the origin of selectivity between \(G_1\) and \(G_2\), i.e., \(G_1\) is more reactive than \(G_1\) in one-electron oxidation of 5'-CG,G,G,-3' sequence.

While molecular oxygen is considered to attack on C5 of the guanine neutral radical as shown in Scheme 1,\(^{28,29}\) the spin density on the C5 positions of the 5'-CO,G,O,-3' state is much larger than that of 5'-CG,G,G,-3' state as shown in Fig. 4. The high reactivity of the 5'-CG,G,G,-3' state cannot be solely explained by superdelocalizability derived from the frontier-electron theory for the corresponding radical orbital, and other explanation is apparently necessary. Probably, the reaction of \(G_1\) radical orbital with molecular oxygen may proceed with a smaller activation energy compared with that of \(G_1\) radical orbital. The transition state for the oxygen adduct formation ultimately leading to imidazolone product (diz) should be explicitly found to elucidate the discrepancy of the reactivity between the 5'-CGGG-3' and 5'-CGGG-3' states.

Figure 6 shows theoretical values of the atomic spin densities for the deprotonated neutral guanine radical in monomer. The atomic spin densities shown in Fig. 6 are very similar to those of the 5'-CGGG-3' (d) and 5'-TGGG-3' radicals (a) and (b) shown in Fig. 3, indicating that the electronic structures of \(G_1\) radicals in the 5'-CGGG-3' and (5'-TGGG-3') radicals behave very similarly as monomer \(G_1\) even in the DNA duplex. On the contrary, it is apparent that the \(G_1\) radical in the 5'-CG,G,G,-3' state exists in an unique interacting environment compared with other \(G_1\)-radicals as depicted in Fig. 4(c). It is, therefore, concluded that the unique and high reactivity of the \(G_1\) of 5'-CG,G,G,-3' is induced by the strong stacking interaction with 5'-side G of the opposite strand.

Figure 6. Spin densities of deprotonated neutral guanine radical in monomer at HF/6-31G level.

Conclusion

The selectivites for photoinduced one-electron oxidation of 5'-TGGG-3' and 5'-CGGG-3' have been examined experimentally and by ab initio MO calculations. Figure 5 shows the summary of the selectivites of the initial stages of one-electron oxidation of both the 5'-TGGG-3' and 5'-CGGG-3' sequences.

Our conclusions are summarized as follows.

(1) Although all 5'-G,G,-3' sequences in B-form DNA have high \(G_1\) selectivity for one-electron
oxidation, 5′-G1G2G3-3′ triplets have G2 selectivity in the cases of 5′-TG1G2G3T-3′ and 5′-AG1G2G3A-3′ sequences. The spin densities of G1′ and G2′ have similar pattern in each other and the shapes of the radical orbitals are also similar. The selectivity is primarily due to the stability of the (5′-G1G2G3-3′) neutral radicals, that is, the 5′-G1G2G3-3′ is more stable in energy than the 5′-G1G2G3-3′. The large spin densities on C5s of 5′-TG1°G2G3-3′ and 5′-TG1OG2G3-3′ are also consistent with the fact that molecular oxygen attack preferentially on this position to give imidazolone product (dlz).

The 5′-CG1G2G3-3′ sequence is very unique with high G1 selectivity. The spin density on N1 of G1′ is distinguishably different from the corresponding spin density of G2′ which has a similar pattern to those of G1′ and G2′ in 5′-TG1G2G3-3′ triplets. While the radical orbital (SOMO) of G1′ is delocalized up to the paired cytosine base, the radical orbital of G2′ is essentially localized on guanine base. The unique electron population of 5′-CG1°G2G3-3′ is originated from the stacking interaction with 5′-side G of the opposite strand. This drastic difference of the electron population in the radical orbitals can explain why G1 is more reactive than G2.

The radical orbitals (SOMOs) in states of radical cations and neutral radicals of 5′-TGGG-3′ and 5′-CGGG-3′ are not the highest occupied molecular orbitals (HOMO). The radical orbitals of the radical cations are more stabilized than those of the radicals.

(4) The selectivity of the oxidation is not determined solely by the stability of radical cations and/or neutral radicals. The electronic structures corresponding to the radical orbitals may play a very important role for the selectivity.

The guanine is most easily oxidized among the nucleobases, and the GGG triplets are most effective. However, as elucidated in this work, the guanine base is drastically affected by the interaction fields induced by base pairing and base stacking. These effects have naturally to be taken into account in order to explain the reactivity and the selectivity in one-electron oxidation of GGG triplets. The electron-loss center created in duplex DNA ultimately moves to end up at G residue via hole migration through the DNA duplex. It may not be thought as a sole pathway that the hole passes through the stacked bases due to the overlap of π-electrons of the stacked bases.

Experimental Sections

General: Riboflavin (1) was purchased from Nacalai Tesque Co., Ltd., and cyano substituted benzophenone 2 was synthesized as reported previously.12 Calf thymus DNA was purchased from Pharmacia Biotech. The oligodeoxynucleotides d(CGTA CTCTTTGCTGGG-CTTGGTTTCTTTCTT) (33-mer), its complementary strand d(AAAGAAAGAAACCCAAGCC CGAACCAGAGTACG) (33-mer), d(CATTCCGGCTTG) (12-mer), and its complementary strand d(CAA GCCGAAATG) (12-mer) were purchased from Greiner Japan Co. Ltd. The oligodeoxynucleotide d(ATGGGT ACCCAT) was purchased from NIPPON GENE (10 units/μL) and [γ-32P]-ATP (10 nCi/mL) was from Amersham. All aqueous solutions utilized purified water (MILLIPORE, Mili-Q sp UF). Photolysis at 366 nm was carried out using a COSMO BIO CSF-20AF transilluminator. Photolysis at 312 nm was carried out using a VILBER LOURMAT TFX-20M transilluminator. A Gibco BRL Model S2 sequencing gel electrophoresis apparatus were used for polyacrylamide gel electrophoresis (PAGE). The gels were analyzed by autoradiography with a BIORAD Model GS-700 Imaging densitometer and BIORAD Molecular Analyst software (version 2.1). CD spectra were recorded on a JASCO J-720 spectropolarimeter.

Preparation of 5′-32P-End-Labeled ODN

The oligonucleotide 33-mer (400 pmol strand concentration) was 5′-end-labeled by phosphorylation with 4 μL of [γ-32P]-ATP and 4 μL of T4 polynucleotide kinase using standard procedures.19 The 5′-end labeled oligonucleotides were recovered by ethanol precipitation and further purified by 15% preparative non-denaturing gel electrophoresis and isolated by the crush and soak method.20

Cleavage of 5′-32P-End-Labeled ODNs by Photolysis in the Presence of Photosensitizer
5'-32P-end-labeled ODN 33-mer was hybridized to the complementary strand (2.5 μM, strand concentration) in 10 mM sodium cacodylate buffer, pH=7.0, without salts such as NaCl. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The 5'-32P-end-labeled ODN duplex (2.0 × 10^6 cpm) containing a photosensitizer and calf thymus DNA (10 μM, base concentration) was irradiated with a transilluminator at 4 °C for 40 min. After irradiation, all reaction mixtures were ethanol-precipitated with the addition of 10 μL of 3 M sodium acetate and 800 μL of ethanol. The precipitated DNA was washed with 100 μL of 80% cold ethanol and dried in vacuo. The dried DNA was dissolved in 50 μL of water or 10% piperidine (v/v), heated at 90 °C for 20 min, evaporated by vacuum rotary evaporation to dryness and resuspended in 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). All reactions, along with Maxam-Gilbert G+A and C+ T sequencing reactions, were heat-denatured at 90 °C for 3 min and quickly chilled on ice. The samples (1-2 μL, 2-5 × 10^3 cpm) were loaded onto 12% polyacrylamide sequencing gels and electrophoresed at 1900 V for approximately 2 h and transferred to a cassette and stored at -80 °C with Fuji X-ray film (RX-U). The gels were analyzed by autoradiography with a densitometer and BIORAD Molecular Analyst software (version 2.1). The intensities of the spots resulting from piperidine treatment were determined by volume integration.

References


5. For a review, see Burrows, C. J.; Muller, J. G. Chem. Rev. 1998, 98, 1109.


Chapter 3

Mapping of Highest Occupied Molecular Orbitals of Duplex DNA by Cobalt-Mediated Guanine Oxidation

Abstract: I have examined the oxidation of oligodeoxynucleotides (ODNs) containing various guanine (G)-containing sequences with Co(ll) ion and benzoyl peroxide (BPO). Sequence dependent G-cleavage has been observed for double-stranded ODN as revealed by PAGE analysis of the reaction mixture after hot piperidine treatment, whereas non-selective equal G cleavage was observed for single stranded ODN. The relative rates of sequence dependent G oxidation were determined by densitometric assay of the DNA cleavage bands. I then performed ab initio calculations of HOMOs of the G-containing sequences with B-form geometry at the HF/6-31G* level. Experimentally observed relative rates of G oxidation matched well with the calculated HOMOs of the G-containing sequences. Thus, the DNA cleavage data obtained from the oxidation of duplex ODNs with Co(ll) ion in the presence of BPO correlated nicely with calculated HOMOs, implying that the Co(ll) ion is coordinated more strongly to the G having a larger HOMO. These results suggest that the coordination of Co(ll) ion to the N7 of guanine base in a duplex DNA is a HOMO-controlled process, in accordance with the previous NMR studies on the sequence dependent binding of Co(ll) ion to the N, of G residues of duplex ODN. After performing ab initio calculations of a number of G-containing sequences, I found important general trends that represent the distribution of HOMOs of G bases in B-form DNA. The results of HOMO mapping described here are extremely important for predicting: (i) which G-sites are more susceptible to electrophilic attack in chemical and biological reactions, such as DNA alkylation by antitumor drugs or mutagens, and (ii) which G-sites are more prone to HOMO-LUMO interactions with DNA binding drugs and proteins. These results provide a new tool for probing the heterogeneity of DNA sequences.
Introduction

The highest occupied molecular orbital (HOMO) of organic molecules plays a crucial role in chemical reactions by interacting with the lowest unoccupied molecular orbital (LUMO) of reactant molecules. Despite enormous contribution of HOMOs of small molecules in organic chemistry, the HOMOs of naturally occurring macromolecules such as DNA and RNA have not been well recognized, because the calculation of the HOMOs of such large molecules like duplex DNA is very difficult and, as a consequence, experimental mapping of the HOMO of duplex DNA has not been undertaken. Consequently, there has been a lack of appreciation for the role HOMO-LUMO interactions play in DNA-drug and DNA-protein interactions, although HOMO-LUMO interactions between monomer guanine base and alkylating agents or intercalators have been reported.

Theoretical calculations of DNA bases have been extensive for ionization potentials of monomeric nucleobases, stability of nucleobase-pair in neutral and radical cation states, and for stacking interactions between nucleobases. However, only a few ab initio calculations on stacked nucleobases have been reported. Several years ago, we demonstrated experimentally and by theoretical calculations, that the HOMO of a stacked 5'-GG-3' sequence was largely localized on the 5'-G of the GG doublet, with no HOMO at the single G (underlined). In contrast, in another duplex (5' -TCGGT-3'/3'-AGCCA-5'; Figure 1b), the HOMO distribution at the single G (underlined) was considerably large. The only difference between these duplexes was that the GG doublet was flanked on either the 5' side or the 3' side by a G-C base pair as illustrated in Figure 2a. Thus, the ab initio calculations clearly indicated that the HOMO localization on G base is highly sequence dependent.

General Trends for the HOMO Distributions in B-Form DNA

After performing ab initio calculations of a number of G-containing sequences, I found important general trends that represent the distribution of HOMOs of G bases in B-form DNA. First, the HOMOs of stacked GG doublets are localized overwhelmingly on the 5'-G, regardless of the 3'- and 5'-flanking sequences (A, C or T). Second, in stacked GGG triplets (Figures 2b,e), the HOMO distribution is always greater for the 5'-G than the middle G with almost no HOMO at the 3'-G, regardless of the 3'- and 5'-flanking sequences (A, C or T). Another important feature is that the single Gs of 5'-CCG-3' sequences have exceptionally

Results and Discussion

HOMOs of G-Containing DNA Sequences Obtained by Ab Initio Calculations

I performed ab initio calculations of HOMOs for a wide variety of G-containing sequences with B-form geometry using GAUSSIAN 94 at the HF/6-31G* level. Double strands of B-form 5-mers containing a variety of G sequences were built using the Insight II program with standard B-form parameters. For the quantum mechanical studies, all of the sugar backbones of the duplex 5-mers were removed from the coordinate file (whilst keeping the positions of all of the atoms fixed) and replaced by methyl groups. The orbital contour plots of the calculated HOMOs of typical duplex 5-mers are shown in Figure 1, and the distributions of HOMOs are summarized in Figure 2. As seen in Figure 1a, the HOMO of the duplex 5'-TGGCT-3'/3'-ACCGA-5' sequence was largely localized on the 5'-G of the GG doublet, with no HOMO at the single G (underlined). In contrast, in another duplex (5'-TCGGT-3'/3'-AGCCA-5'; Figure 1b), the HOMO distribution at the single G (underlined) was considerably large. The only difference between these duplexes was that the GG doublet was flanked on either the 5' side or the 3' side by a G-C base pair as illustrated in Figure 2a. Thus, the ab initio calculations clearly indicated that the HOMO localization on G base is highly sequence dependent.

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large HOMOs compared with the single Gs of other sequences (Figure 1b). It should be noted that in 5'-CCG-3' sequences, the single G (underlined) is strongly stacked with the 5'-G of the GG doublet on the opposite strand (Figures 2a,d). A similar feature has also been observed on the extraordinary high HOMO energies of 5'-CGG-3'/3'-GCC-5' sequence compared with those of other GG doublets due to the strong stacking of the single G (underlined) with the 5'G of the GG doublet on the opposite strand.6-9 The calculated HOMOs of the G-containing sequences shown in Figure 2 predict the relative susceptibility of each G toward electrophilic attack and HOMO-LUMO interactions with the LUMO of DNA-binding molecules.

Attempts for Experimental HOMO Mapping

I have then attempted an experimental mapping of the HOMO of duplex DNA. As a mapping probe, I used an oligodeoxynucleotide (ODN) containing 5'-TGGTT-3', 5'-CGGGC-3' and 5'-TGGGT-3' sequences (ODN 1; Figure 3). In one-electron oxidation such as riboflavin-sensitized photooxidation, the 5'-Gs of 5'-TGGT-3' and 5'-CGGGC-3' sequences have been known to be selectively oxidized,6-9,11 while 5'-TG,G,G,T-3' was preferentially oxidized at G1, as shown in Figure 3a.6-9,11 These results, particularly those of 5'-TGGGT-3' sequence are not in agreement with the calculated HOMOs (cf. Figure 2b vs.
Figure 3a). In contrast, recent $^1$H NMR studies of the coordination of the Co(II) ion to the N$_7$ of G in DNA oligomers, have shown that the binding selectivity of the Co(II) ion towards 5'-TG$_1$G$_2$G$_3$T-3' sequence follows the order G$_1$ > G$_2$ > G$_3$ while holding a high 5'-G selectivity for GG doublets. These binding selectivities are quite compatible with the calculated HOMOs of Figure 2b. Crystallographic studies have indicated that the Co(II) ion binds exclusively to the N$_7$ of G by coordination. I, therefore, assumed that the coordination of low concentrations of Co (II) ion, a soft Lewis acid, to the N$_7$ of electron rich G is a HOMO controlled process and, as a consequence, the sequence selectivity of the Co (II) ion binding obtained from the $^1$H NMR studies would directly reflect the HOMO distribution in G-containing sequences.

Comparison of Cobalt-Mediated G-Oxidation with Calculated HOMO

Previously, Co(II) ion has been shown to mediate G oxidation in the presence of oxidants such as HSO$_5^-$ to produce piperidine labile sites. It has also been reported that sequence selective G cleavage was observed in the oxidation of DNA with BPO in the presence of CuCl. With these in mind, I performed DNA cleavage experiments in the presence of low concentrations of Co(II) ion, using benzoyl peroxide (BPO) as an oxidant. Incubation of the double-stranded 5'-P-5'-end-labeled ODN 1/2 with Co(II) ion (such as CoCl$_2$ or Co(OAc)$_2$) and BPO at 37 °C for 5 min, followed by treatment with hot piperidine, resulted in a highly selective G cleavage (Figure 4, lanes 1 and 2). The degree of cleavage was then determined using a densitometric assay. For direct comparison with the calculated HOMO data, the cleavage data was normalized by assigning a value of 100 to the largest cleavage band in each segment (as indicated by the block shown in Figure 3). The DNA cleavage data of duplex ODN 1/2 was in complete agreement with the calculated HOMOs (cf. Figure 2b vs. Figure 3b). In contrast, using identical conditions to the ODN 1/2 cleavage reaction, the single stranded ODN 1 without ODN 2 showed non-selective G cleavage (lanes 3 and 4).

It has been reported that a high concentration of Co(II) ion induced a conformational change of B-form DNA containing G-rich sequences. In order to confirm whether Co(II) ion induces a conformation change of these G-containing duplex ODN under the reaction conditions, we measured the CD spectrum of a duplex 5'-CATTTCGGGCTTG-3'/5'-CAAGCCCGAAATG-3'. However, no conformational change has been observed upon addition of CoCl$_2$ up to 200 μM (20 eq to ODN) under the DNA cleavage reaction conditions as shown in Figure 5.

Next, I examined the HOMO mapping of other duplexes ODN 3/4 and ODN 5/6 by comparing the reactivity of 5'-CCG-3' sequence possessing a larger HOMO with that of 5'-GCC-3' sequence using a GG doublet as an internal standard. As shown in Figure 6 (lanes 1 and 8), the 5'-CCG-3' sequence had a much higher reactivity than the 5'-GCC-3' sequence, as predicted from the calculated HOMOs (Figure 2a). The DNA cleavage data shown in Figure 6 are again in good agreement with the calculated HOMOs. I also examined the...
HOMO mapping of other duplexes and the DNA cleavage data were again in good agreement with the calculated HOMOs (Figure 7–9).

\[ \text{CoCl}_2 \]
\[ \text{Co(OAc)}_2 \]
\[ \text{BPO} \]
\[ \text{piperidine treatment} \]

Figure 4. Autoradiogram of the denaturing gel of the \( ^{32} \text{P}-5' \)-end-labeled duplex ODN 1/2 after incubation with BPO in the presence of cobalt ion. \( ^{32} \text{P}-5' \)-end-labeled ODN 1 was hybridized to the complementary ODN 2 and the duplex was incubated with BPO and cobalt ion as described in the experimental section.

Figure 5. CD spectra of 5'-CATTTCGGCTTG-3' / 5'-CAAGCCCGAAATG-3' (10 \( \mu \text{M}, \) strand concentration) in 10 mM sodium cacodylate buffer (pH 7.0) and NaCl 100 mM at 37 °C. Solid line is in the presence of Co(II) (200 \( \mu \text{M} \)) and dotted line is in the absence of Co(II).

Figure 6. Autoradiogram of the denaturing gel of the \( ^{32} \text{P}-5' \)-end-labeled duplex ODNs after incubation with BPO in the presence of cobalt ion. \( ^{32} \text{P}-5' \)-end-labeled ODN 3 and \( ^{32} \text{P}-5' \)-end-labeled ODN 5 were hybridized to ODN 4 and ODN 6, respectively. The \( ^{32} \text{P}-5' \)-end-labelled duplexes were incubated with BPO (10 \( \mu \text{M}, \) final conc) in the presence of cobalt ion (3 \( \mu \text{M}, \) final conc) as described in the experimental section.
Figure 7. (a) Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the 32P-5' end-labeled ODN 25-mer (Co2+ and BPO) sequence after incubation of the duplex in the presence of cobalt ion and benzoyl peroxide (BPO) at 37 °C. 32P-5'-end-labeled ODN 25-mer was hybridized to the complementary strand (2.5 μM, strand concentration) in 10 mM sodium cacodylate buffer, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing cobalt ion (15 μM) and BPO (50 μM) was incubated at 37 °C for 5 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (b) Intensities of the DNA cleavage bands. The intensities were normalized by assigning a value of 100 to the largest cleavage band in each segment (as indicated by the block). The arrows indicate the cleavage sites. (c) Distribution of HOMOs obtained by ab initio HF6-31G* single-point calculations. The arrows indicate the distribution of the HOMOs normalized with the largest HOMO as 100.

Figure 8. (a) Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the 32P-5'-end-labeled ODN 25-mer (Co2+ and BPO) sequence after incubation of the duplex in the presence of cobalt ion and benzoyl peroxide (BPO) at 37 °C. 32P-5'-end-labeled ODN 25-mer was hybridized to the complementary strand (2.5 μM, strand concentration) in 10 mM sodium cacodylate buffer, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing cobalt ion (15 μM) and BPO (50 μM) was incubated at 37 °C for 5 min. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (b) Intensities of the DNA cleavage bands. The intensities were normalized by assigning a value of 100 to the largest cleavage band in each segment (as indicated by the block). The arrows indicate the cleavage sites. (c) Distribution of HOMOs obtained by ab initio HF6-31G* single-point calculations. The arrows indicate the distribution of the HOMOs normalized with the largest HOMO as 100.
Figure 9. (a) Autoradiogram of a denaturing 12% polyacrylamide/7 M urea gel for the 5'-AT AA T AACAAGCCAACCGAT AT ACG-3' sequence after incubation of the duplex in the presence of cobalt
ion and benzoyl peroxide (BPO) at 37°C. 32 P-5'-end-labeled ODN 25-mer was hybridized to the complementary
strand (2.5 pM, strand concentration) in 10 mM sodium cacodylate buffer, pH 7.0. Hybridization was achieved
by heating the sample at 90°C for 5 min and slowly cooling to room temperature. The 32 P-5'-end-labeled ODN
duplex (2.0 × 10^5 cpm) containing cobalt ion (10 pM) and BPO (50 pM) was incubated at 37°C for 2 min.
After piperidine treatment (90°C, 20 min), the sample was dried and electrophoresed through a denaturing
12% polyacrylamide/7 M urea gel. (b) Intensities of the DNA cleavage bands. The intensities were normalized by
assigning a value of 100 to the largest cleavage band of 5'-CCGA-3'. The arrows indicate the cleavage sites.

While the DNA cleavage data experimentally obtained are fully consistent with the hypothesis that Co(II) ion is coordinated more strongly to the guanine N_7 having a larger HOMO, there seems to be an alternative explanation. Positively charged cobalt ion would bind to the most negative G sites in duplex DNA via an electrostatic interaction. I, therefore,
examined the ab initio calculations of molecular electrostatic potentials (MEPs) for two
duplexes, 5'-TCGG-3'/5'-CCGA-3' and 5'-TGCC-3'/5'-GCCA-3'. The MEPs were
calculated at the HF/6-31G* level and compared with the calculated HOMOs of the same
duplexes. In the electrostatic potential map of Figure 10b, pink and blue indicate negative
electrostatic potential. In both duplexes, the difference of the negative electrostatic potentials
between the 5'-G and the 3'-G of GG doublet was not so large as observed for HOMO,
inconsistent with the experimental results (cf. Figure 10b vs. 10a). I also performed ab initio
calculations of the total atomic charges of all guanine N_7 for the two duplexes at the HF/6-31G* level. As shown in Figure 10c, the total atomic charge of N_7 (-0.55405) for the single
G (underlined) of 5'-CCGA-3' sequence of duplex 5'-TCGG-3'/5'-CCGA-3' was much
larger than that of the 5'-G (-0.55047) of GG doublet, quite inconsistent with the
experimental results. Thus, the calculated atomic charges and Pullman's MEPs cannot fully
explain the results of ODN 3/4 and ODN 5/6 shown in Figure 3b, although sequence
selective guanine N_7 alkylation of contiguous Gs by positively charged DNA alkylating agents
has been explained reasonably by MEPs. All the experimental results are best explained by
calculated HOMOs, i.e., the experimental data obtained by the CoCl_2-BPO reaction are
consistent with the assumption that cobalt ion binds most strongly to the G having a largest
HOMO, although there is no direct proof to support that G oxidation is a HOMO controlled
reaction.

In order to get insight into the mechanism of the G oxidation, I analyzed the reaction
mixture obtained from the incubation (37°C, 2.5 h) of self-complementary ODN, 5'-
ATGGGTACCCAT-3' with CoCl_2 and BPO under the same conditions as those for DNA
cleavage experiments followed by enzymatic digestion. Due to the complexity of the reaction
mixture together with low conversion of the ODN, attempts to isolate the oxidation products
were unsuccessful. However, by the HPLC analysis, the formation of a small amount of
7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) was observed by comparison of the HPLC
retention time and the UV spectrum obtained by photodiode array assay with those of authentic
sample (Figure 11). It should be noted that 8-oxo-dG was produced in the oxidation of DNA
with CuCl and BPO.16b
Figure 10. Comparison of orbital contour plot of HOMOs (a), molecular electrostatic potential (MEP) maps (b), and total atomic charges of guanine N7 (c) for two duplexes, 5'-TCGG-3'/5'-CCGA-3' and 5'-TGGC-3'/5'-GCCA-3'. (a) Ab initio calculations at the HF/3-21G level. The sequences are shown on both sides. The sugar backbones were replaced by methyl groups. The values on both sides indicate the distribution of the HOMOs normalized to the largest HOMO as 100. (b) In the electrostatic potential map, pink and blue indicate negative electrostatic potentials, and yellow and green represent positive electrostatic potentials. (c) Total atomic charges of guanine N7 (pink sphere). The sequences are shown on both sides. The sugar backbones were replaced by methyl groups. The values were calculated at the HF/6-31G* level (red).

Figure 11. HPLC analysis of nucleosides produced by enzymatic digestion of damaged oligonucleotides. The duplex 5'-ATGGGTACCCAT-3' (self-complementary, 1 mM base concentration) hybridized in 10 mM sodium cacodylate buffer pH 7.0 and 100 mM NaCl. was incubated in the presence of Co(II) (100 μM) and benzoyl peroxide (BPO; 500 μM) at 37 °C for 2.5 h. After ethanol precipitation, the ODN was subjected to enzymatic digestion and analyzed by HPLC (λ<sub>max</sub>=254 nm). Inset: UV spectra of the arrow peak (solid lime) and 8-oxo dG standard (dotted line).

Conclusion

I was able to demonstrate for the first time the experimental HOMO mapping of duplex DNA using cobalt ions and benzoyl peroxide, in combination with ab initio calculations of a wide variety of G-containing DNA sequences. The simple HOMO mapping method described in this study may allow its application to other nucleic acids, such as different forms of DNA, DNA-RNA hybrids and RNA. The HOMOs shown in Figure 2 provide a simple way of predicting the sequence dependent reactivity of G bases in duplex DNA and their susceptibility to HOMO-LUMO or charge-transfer interactions with electron accepting functional groups of DNA-binding drugs and proteins. While noncovalent intermolecular forces such as electrostatic interactions, stacking interactions, hydrogen bonding, and hydrophobic effects are well-known for DNA-ligand and DNA-protein interactions, the interaction of DNA HOMOs with the LUMOs of DNA-binding molecules could also be
another important binding force. The present HOMO mapping method can visualize the susceptibility of G-containing sequences to HOMO-LUMO interactions with DNA-binding molecules including transition metal ions, thus providing a new tool for probing the heterogeneity of DNA sequences. These results have led to a new view of the chemical and physicochemical properties of DNA and the sequence dependent reactions of G bases in DNA. The consequence of HOMO-LUMO interactions in metal-DNA, drug-DNA or protein-DNA interactions is clearly an important area to be investigated in future studies.

Experimental Section

General: Cobalt chloride (CoCl₂) was purchased from Nacalai Tesque Co., Ltd. Benzoyl peroxide (BPO) was purchased from Wako Pure Chemical Industries, Ltd. Calf thymus DNA (1 g) was purchased from Pharmacia Biotech. The oligodeoxynucleotides were purchased from Pharmacia Biotech. T4 kinase was purchased from NIPPON GENE (10 units/μL) and γ-[³²P]-ATP (10 mCi/ml) was from Amersham. All aqueous solutions utilized purified water (MILLIPORE, Mili-Q sp UF). A GIBCO BRL Model S2 sequencing gel electrophoresis apparatus was used for polyacrylamide gel electrophoresis (PAGE).

Theoretical Calculations

Ab initio calculations were performed at the HF/3-21G level for molecular electrostatic potentials (MEPs) and at the HF/6-31G* level for other calculations utilizing the GAUSSIAN 94 program package. Geometries of the duplex consisting of stacked methylated nucleobases at N₁ (pyrimidine base) and N₉ (purine base) were constructed as follows. The corresponding duplex 4-mers or 5-mers were built using the insight II program (Version 97.0) with standard B-form helical parameters (pitch, 3.38 Å; twist, 36°; tilt, 1°) which have been optimized by X-ray crystallographic analysis of relevant monomers and X-ray diffraction data of polymers. All the sugar backbones of the duplex were removed except for the deoxyribose C1’ carbon and C1’ H and two H atoms were then attached to the C1’ methine to complete stacked N-methylated nucleobases, keeping the position of all atoms fixed. HOMOs of the calculated 5-mers were displayed graphically using Gaussian1/F. MEPs were displayed graphically using Cerius² (Version 3.8).
Preparation of $5'$-$32$P-End-Labeled ODN

The oligonucleotides (ODNs, 400 pmol strand concentration) were $5'$-end-labeled by phosphorylation with 4 μL of $[^\gamma-32P]ATP$ and 4 μL of T4 polynucleotide kinase using standard procedures. The $5'$-end-labeled oligonucleotides were recovered by ethanol precipitation and further purified by 15% preparative nondenaturing gel electrophoresis and isolated by the crush and soak method.

DNA Cleavage Experiments Using Cobalt Ion and BPO

The $32$P-end-labeled ODNs were hybridized to their complementary ODNs (2.5 μM, strand concentration) in 10 mM sodium cacodylate buffer (pH 7.0). Hybridization was achieved by heating the samples to 90°C for 5 min and then slowly cooling the samples to room temperature. The $32$P-end-labeled duplex solutions were added to a 1.5 mL Eppendorf tube containing 35 μL of MilliQ water, 20 μL of 50 μM aqueous cobalt chloride or 50 μM cobalt acetate solution (15 μM, final conc), and 5 μL of freshly prepared 1 mM BPO solution in acetonitrile (50 μM, final conc). After incubation at 37 °C for 5 min, the solutions were diluted with 10 μL of calf thymus DNA (50 μM, base pair conc) and 10 μL of 3 M sodium acetate buffer (pH 5.2). After precipitation with cold ethanol (800 μL) for 20 min at −80 °C followed by centrifugation (15 min at 0 °C, 1.5 $\times$ 10$^4$ rpm), the resulting DNA pellets were washed with cold 80% ethanol and dried under vacuum using a Speedvac. After the DNA pellets were treated with 1 M piperidine for 20 min at 90 °C, the solution was evaporated under vacuum, and the piperidine was coevaporated with water twice. The radioactivity of the samples was then assayed using an Aloka 1000 liquid scintillation counter, and the dried samples were dissolved in 80% formamide loading buffer (to reach a radiation density of 2000-3000 cpm/μL) and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel (1900 V, 1.5 h). The gels were exposed to X-ray film with an intensifying screen at −70 °C. The degree of DNA cleavage was assayed using a BIORAD Model GS-700 Imaging densitometer and BIORAD Molecular Analyst software (Version 2.1).

CD Measurement

CD spectra were measured on a JASCO J-720 spectropolarimeter. The ODNs (10 μM, strand concentration) were hybridized in 10 mM sodium cacodylate buffer (pH 7.0) and 100 mM NaCl. Hybridization was achieved by heating the sample to 90 °C for 5 min and then slowly cooling the sample to room temperature. Aqueous cobalt chloride (200 μM, final concentration) was added before CD measurement. The sample solution was measured at 37 °C. CD data were transformed into molar ellipticity [θ] in the units of degree cm$^2$/dm of monomer subunits.

Product Analysis

Self complementary ODN 5'-ATGGGTACCCAT-3' (1 mM base concentration, 83 μM strand concentration) was hybridized in 10 mM sodium cacodylate buffer pH 7.0 and 100 mM NaCl. Hybridization was achieved by heating the samples to 90 °C for 5 min and then slowly cooling the samples to room temperature. The 12-mer ODN was added to a 1.5 mL Eppendorf tube containing MilliQ water, 1 mM aqueous cobalt chloride (100 μM, final concentration), and freshly prepared 5 mM BPO solution in acetonitrile (500 μM, final concentration). After incubation at 37 °C for 2.5 h, the solution was diluted with 20 μL of 3 M sodium acetate buffer (pH 5.2). After precipitation with cold ethanol (800 μL) for 1 h at −80 °C followed by centrifugation (15 min at 0 °C, 1.5 $\times$ 10$^4$ rpm), the resulting DNA pellets
were dried under vacuum using a Speedvac. The ODN was then subjected to enzymatic digestion with alkaline phosphatase (1000 U/ml) and nuclease P1 (1000 U/ml) and phosphodiesterase I (3 U/ml) at 37 °C for 2 h. DNA oxidation products were analyzed on a CHEMCOBOND 5-ODS-H column (4.6 x 150 mm) by reversed-phase HPLC on a GILSON programal pump Model 305 detected at 254 nm. Elution was with a mixture of 50 mM triethylammonium acetate, 0% acetonitrile (isocratic)/5 min, 0–7% 5–27 min, and 7% (isocratic)/27–30 min at a flow rate of 1.0 mL/min. UV spectra were recorded on an OTSUKA ELECTRONICS MCPD-3600 photodiodearray.

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Abstract: In order to investigate whether Co(II) and benzoyl peroxide (BPO) oxidation is able to map the HOMO of DNA possessing other structures than B-form, I have examined the oxidation of oligodeoxynucleotides (ODNs) containing GG and GGG sequences with Co(II) and BPO. Sequence dependent G-cleavage has been observed for DNA-RNA hybrid and DNA quadruplex as revealed by PAGE analysis of the reaction mixture after hot piperidine treatment. The relative rates of sequence dependent G oxidation were determined by densitometric assay of the DNA cleavage bands. Moreover, ab initio calculations of the HOMOs of the G-containing sequences with DNA-RNA hybrid geometry were performed at the HF 6-31G* level. The DNA cleavage pattern had a very similar tendency to the calculated HOMOs of the G-containing sequences, although the experimental data did not match well with the calculated HOMOs. Toward quadruplex DNA, only the 5'-G of GGG or GGGG in quadruplex DNA was selectively cleaved unexpectedly. The 5'-G selective cleavage was also seen in one-electron oxidation by riboflavin under photoirradiation. These results show important properties of quadruplex DNA.
Introduction

As for flexible nature as a biopolymer, DNA is able to form a number of local conformations. In addition to canonical right handed B-form DNA, DNA has been demonstrated to adopt A-form DNA, left handed Z-form DNA, cruciform, parallel stranded DNA, bent DNA, triplex and quadruplex DNA. Such polymorphism of DNA structure has been suggested to play an important role in a number of transcriptional and replicative processes.

For examples, DNA-RNA hybrids occur in several important biological processes. They are intermediates in transcription, in DNA replication, and in the synthesis of retroviral cDNA by reverse transcription. Ribonuclease H (RNase H), from Escherichia coli recognizes the DNA-RNA hybrid duplex as its substrate and hydrolyzes the phosphodiester linkages in the RNA strand. The reverse transcriptase of human immunodeficiency virus, which synthesizes the proviral DNA, using its RNA as a template, contains an RNase H domain that is structurally similar to E. coli RNase H. These enzymes recognize only DNA-RNA hybrid duplexes, and not DNA or RNA duplex. Although the mechanism of the substrate specificity of RNase H has not yet been determined, the tertiary structure of the hybrid duplex is assumed to determine the specificity. DNA-RNA hybrids are also exploited in antisense drug technology.

Telomeres are important multifunctional nucleoprotein structures found at the end of eukaryotic chromosomes. They ensure the complete replication of chromosomal DNA and protect the chromosome ends from fusion and degradation. In vertebrates, telomeric DNA contains a double-stranded region made from the simple repeat sequence TTAGGG and a single-stranded 3' -end overhang. The single-stranded tail of human telomeres is surprisingly long, averaging more than 150 bases in length, and may be involved in controlling telomerase activity and telomere to telomere interactions. Telomeres of human somatic cells have 1000–3000 repeats and gradually shorten with every cell division. This shortening serves as a mitotic clock that appear to limit the proliferative capacity of somatic cells. In contrast, immortal cancer cells have an unlimited proliferative potential, and as a prerequisite, they must maintain stable telomeric length. The major mechanism of telomere length maintenance is the reactivation of telomerase, a specific reverse transcriptase with an endogenous RNA template. A high level of telomerase activity has been associated with cancer cells and may be essential for their immortality. Therefore, telomerase and in a broader sense, the telomere itself, has been proposed as a potential chemotherapeutic target for nontoxic anticancer agents.

In chapter 3, I suggested that there seems to be important binding force, the interaction of the HOMO of duplex DNA with the LUMO of DNA binding molecules such as drugs and protein, and reported the first experimental HOMO mapping of duplex DNA using Co(II) and benzoyl peroxide (BPO), in combination with ab initio calculations of various guanine (G)-containing DNA sequences. If we are able to know the HOMO location of special DNA structures mentioned above, very important and useful views on the elucidation of recognition mechanism such as transcriptional process might be obtained. Thus, we examined the experimental HOMO mapping of DNA-RNA hybrid (A-form like DNA) and quadruplex using Co(II) and BPO to investigate whether this Co(II) and BPO oxidation is available for probing DNA conformations other than normal B-form DNA.

Results and Discussion

Ab Initio Calculation of DNA–RNA Hybrid

At first, ab initio calculations of HOMOs of DNA–RNA hybrid were performed at the HF/6-31G* level using GAUSSIAN94. As mentioned in Chapter 3, double-stranded DNA–RNA hybrid 5-mers containing GG and GGG sequences were built using the Insight II program with standard DNA–RNA hybrid parameters. For the quantum mechanical studies, all of the sugar backbones of the duplex 5-mers were removed from the coordinate file (while keeping the positions of all of the atoms fixed) and replaced by methyl groups. The orbital contour plots of the calculated HOMOs of typical duplex 5-mers are shown in Figure 1, and the distributions of HOMOs are summarized in Figure 2. As seen in Figure 1, the
HOMOs of \(d(5'-TGGTT-3')/r(5'-AACCA-3')\) and \(d(5'-TGGGT-3')/r(5'-ACCCA-3')\) are both localized on the 5'-G with almost no HOMO on the other Gs. About \(d(5'-TGGGT-3')/r(5'-ACCCA-3')\), the calculation result was quite different from the result of B-form. The drastic change of HOMO location might be induced by the enormous change of stacking between guanine and guanine.

**Figure 1.** Orbital contour plot of the HOMOs of two B-form DNA 5-mers (a, c) and two corresponding DNA-RNA hybrid 5-mers (b, d) obtained by ab initio calculation using GAUSSIAN 94 at the HF/6-31G* level. The sequences are shown on both sides. The sugar backbones were replaced by methyl groups.

**Figure 2.** Distribution of HOMOs in DNA-RNA hybrid 5-mers obtained by ab initio HF/6-31G* single-point calculations. The arrows indicate the distribution of the HOMOs normalized the largest HOMO as 100.

**CD Measurement for Confirming of DNA-RNA Hybrid Conformation**

Then, I examined the experimental HOMO mapping of DNA-RNA hybrid, as mentioned in Chapter 3, to compare the experimental results with the ab initio results. As a mapping probe, I used an oligodeoxynucleotide (ODN) containing 5'-TGGTT-3' and 5'-TGGGT-3' sequence and both DNA and RNA were used as the complementary strands (DNA duplex 1/2 and DNA-RNA hybrid 1/3; Figure 3).

**DNA 1:** 5'-CGTTATCATTTGGTTATCATTTGGTTATCTCG-3' (33 mer)
**DNA 2:** 3' -GCATAGTAACCAAATAGTAACCCAATAGTAAGC -5' (33 mer)
**RNA 3:** 3' -GCAAUAGUAACCAAUAGUAACCCAAUAGUAAGC -5' (33 mer)
**DNA 4:** 5'-TTAGGGTTAGGGTTAGGGTTAGGG -3' (24 mer)
**DNA 5:** 5'-TTGGGGTTGGGGTTGGGGTTGGGG -3' (24 mer)

**Figure 3.** Sequences of DNAs or RNA used in this chapter. DNAs 1-3 were used for the study of DNA-DNA or DNA-RNA hybrid and DNAs 4, 5 were used for the study of quadruplex DNA.

Before DNA cleavage experiments, in order to confirm whether DNA-RNA hybrid adopts an A-form-like conformation under the cleavage reaction conditions, I measured the circular dichroism (CD) spectrum of a DNA-RNA hybrid 1/3 (Figure 4). As shown in figure 4, the
conformational change between DNA duplex and DNA-RNA hybrid was observed clearly and DNA-RNA hybrid changed to more A-form-like conformation. Moreover, it has been reported that from high resolution DNA crystal structural data, Co(NH$_3$)$_6$$^{3+}$ binds to the O$_2$/N$_7$ sites of guanine bases at the major groove side of GpG step via hydrogen bonds in A-DNA which is converted B-to A-DNA by Co(NH$_3$)$_6$$^{3+}$.$^{25,26}$ Therefore, it is likely that Co(II) and benzoyl peroxide oxidation (experimental HOMO mapping) is available to DNA-RNA hybrid.

![Figure 4. CD spectra of DNA-RNA hybrid (1/3; solid line) and DNA duplex (1/2; dotted line) (100 μM base concentration) in 10 mM sodium cacodylate buffer, NaCl 100 mM (pH 7.0) at 37 °C.](image)

**Experimental HOMO Mapping of DNA-RNA Hybrid Using Co(II) and BPO**

Incubation of the double-stranded $^{32}$P-5'-end-labeled DNA duplex 1/2 or DNA-RNA hybrid 1/3 with Co(II) ion (CoCl$_2$) and BPO at 37 °C for 5 min, followed by treatment with hot piperidine, resulted in a highly selective G cleavage (Figure 5, lanes 2 and 3). The degree of cleavage was then determined using a densitometric assay. The degree of 5'-GGG-3' cleavage was noticed since it seems likely that there was little difference between B-DNA and DNA-RNA hybrid in 5'-GG-3' step, as shown in ab initio calculations (Figure 1 and 2). For direct comparison with the calculated HOMO data, the cleavage data was normalized by assigning a value of 100 to the largest cleavage band in 5'-TGGGT-3' (Figure 5b). The DNA cleavage data of DNA duplex 1/2 was in agreement with the calculated HOMOs as shown in Chapter 3. The DNA cleavage data of DNA-RNA hybrid 1/3 was more 5'-G of 5'-GGG-3' selective than of DNA duplex 1/2. The DNA cleavage pattern had a very similar tendency to the calculated HOMOs of the G-containing sequences, although the experimental data did not match well with the calculated HOMOs (Figure 2 and 5b). Why was not the DNA cleavage data of DNA-RNA in agreement with the calculated HOMOs completely? That seems to be why all DNA-RNA hybrids do not adopt an A-form in the experimental condition. Practically, Gyi et al. discovered that the stability and structure of DNA-RNA hybrids depend on the arrangement of bases and in particular, hybrid duplexes containing purine-rich RNA strands are more stable and more A-like than hybrids with a pyrimidine-rich RNA strand.$^{27}$ From assessment of CD spectra, Lesnik and Freier$^{28}$ concluded that there is a continuum of hybrid conformers that are intermediate between A and B-forms. In my case, purine content and A•T content in hybrid RNA were 61% and 64%, respectively. In Lesnik and Frerers' case, moderate purine and A•T content in hybrid RNA generated the CD spectra with the major positive bands similar to the average of the corresponding RNA and DNA bands.$^{28}$ Thus, the DNA cleavage data of DNA-RNA hybrid 1/3 might be mixed results of A-form and B-form.

In any case, more 5'-G of 5'-GGG-3' selective cleavage than that of B-form, which reflects the conformational change from DNA duplex to DNA-RNA hybrid, was observed in DNA-RNA hybrid by using Co(II) and BPO oxidation. This result had a very similar tendency to the calculated distribution of HOMOs, although the experimental data did not match well with the calculated HOMOs.
CD Measurement of Quadruplex DNA

Before quadruplex DNA oxidation experiments, I measured the CD spectra of DNA 4 (Figure 3, Human telomeric repeat) and DNA 5 (Figure 3, Tetrahymena telomeric repeat) in the presence of 100 mM KCl (Figure 6). The formation and stability of G quadruplex were found to depend on the presence of Na+ or K+. The CD spectrum of DNA 4 in the presence of K+ showed a positive band around 295 nm with a shoulder around 270 nm, indicating that in K+ solution a major fraction of molecules adopted a G-quartet structure as previously reported. The CD spectrum of DNA 5 also showed the same spectrum as previously reported.

DNA Cleavage Experiments of Quadruplex DNA

Telomeric DNA consists of tandem repeats of G-rich DNA sequences that form structures based on the quadruplex DNA, a cyclic hydrogen-bonded array of four coplanar guanine bases (Figure 7a). There are many studies about quadruplex structures using NMR, X-ray, AFM (Atomic Force Microscopy), UV spectra and CD spectra. Also the interaction between quadruplex DNA and small molecules (ethidium bromide, porphyrin, anthraquinone, fluorenone, perylene, etc.) or proteins (Sgs1 helicase, topoisomerase II, telomerase, etc.) are well studied. But, only a few experiments of guanine oxidation of quadruplex DNA have been reported.

I examined the DNA oxidation experiments for quadruplex DNA using i) the previous reported experimental HOMO mapping method (Co(II) and BPO oxidation) as mentioned above, ii) riboflavin photooxidation and iii) dimethyl sulfate (DMS). DMS footprinting is often used to characterize the secondary structures of the telomere repeats. Since N7 of
guanine is positioned in the major groove of a normal B-form DNA and is not involved in hydrogen bonding between GC base pairs, it can be easily methylated by DMS, leading to DNA strand cleavage upon piperidine treatment. The methylation reaction and subsequent strand cleavage can produce distinct bands corresponding to the reacting guanines when the DNA samples are subjected to gel electrophoresis. In quadruplex DNA involving guanine tetrads, four Hoogsteen base pairs are created (Figure 7a). N7 of each guanine is hydrogen-bonded to N2 of the adjacent guanine and is, therefore, protected from strand cleavage experiments were shown in Figure 8. In the absence of K+, all guanines corresponding to the four TT AGGG repeats appear unprotected from DMS modification, suggesting no involvement of Hoogsteen hydrogen bonding (lane 6). Therefore, guanines in this case did not form quadruplex and formed single strand. Co(II) and BPO lane also showed non-selective G cleavage like DMS (lane 8), which was agreed with the results of DNA duplex shown in Chapter 3. On the other hand, riboflavin scarcely cleaved DNA (lane 7). It seems likely from these results that riboflavin binds to DNA by intercalation or groove binding.

The results of quadruplex DNA cleavage experiments were shown in Figure 8. In the presence of K+ 100 mM, DMS did not cleave any guanine (lane 12), which indicated the formation of quadruplex. Meanwhile, all the 5'-side guanines of the four TTAGGG repeats were cleaved selectively by Co(II) and BPO (lane 10) or by riboflavin (lane 11). The same cleavage patterns of single strand DNA and quadruplex DNA were also seen in other telomeric repeats DNA S (Figure 9). Next, I compared the cleavage patterns of quadruplex DNA with those of duplex DNA in order to confirm whether the 5'-G selective cleavage depends on quadruplex structure (Figure 10). In duplex DNA, DMS cleaved guanines selectively as observed for single strand (lane 6). Both riboflavin and Co(II) and BPO cleaved
5'-G and middle G of the four TTAGGG repeats (lanes 7, 8) and the cleavage patterns were quite different from those of quadruplex (lanes 10, 11).

Figure 9. Autoradiograms of a denaturing gel electrophoresis for 32P-5'-end-labeled ODN 5': TTGGGTTGGGTTGGGTTGGG-3' sequence (DNA 5) after incubation of the duplex in the presence of cobalt ion and benzoyl peroxide (BPO) or dimethyl sulfate (DMS) at 37 °C or after photooxidation of the duplex in the presence of riboflavin. 32P-5'-end-labeled ODN 24-mer was heated at 90 °C for 5 min in 10 mM sodium cacodylate buffer, pH 7.0 and 100 mM KCl and slowly cooling to room temperature for quadruplex formation. The 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing cobalt ion (10 μM) and BPO (50 μM) was incubated at 37 °C for 5 min. The 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing DMS (10 μM) was incubated at 37 °C for 10 min. 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing riboflavin (50 μM) was irradiated with a transilluminator (366 nm) for 40 min at 0 °C. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lanes 1, 17) DNA, dark control, no piperidine treatment; (lanes 2, 16) irradiated DNA without riboflavin; (lanes 3, 15) incubated DNA without drug; (lanes 4, 14) incubated DNA with BPO; (lanes 5, 13) incubated DNA with CoCl2; (lanes 6, 12) incubated DNA with DMS; (lanes 7, 11) irradiated DNA with riboflavin; (lanes 8, 10) incubated DNA in the presence of CoCl2 and BPO; (Lane 9) Maxam–Gilbert sequencing reactions G+A. Lanes 1–8 (10–17) show the result of single strand (quadruplex).

DNA duplex

quadruplex

Figure 10. Autoradiograms of a denaturing gel electrophoresis for 32P-5'-end-labeled ODN 5': TTAGGGTTAGGTAGGTAGGG-3' sequence (DNA 4) after incubation of the duplex in the presence of cobalt ion and benzoyl peroxide (BPO) or dimethyl sulfate (DMS) at 37 °C or after photooxidation of the duplex in the presence of riboflavin. 32P-5'-end-labeled ODN 24-mer was heated at 90 °C for 5 min in 10 mM sodium cacodylate buffer, pH 7.0 and 100 mM KCl and slowly cooling to room temperature for quadruplex formation. The 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing cobalt ion (10 μM) and BPO (50 μM) was incubated at 37 °C for 5 min. The 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing DMS (10 mM) was incubated at 37 °C for 10 min. 32P-5'-end-labeled ODN duplex (2.0 x 10^5 cpm) containing riboflavin (50 μM) was irradiated with a transilluminator (366 nm) for 40 min at 0 °C. After piperidine treatment (90 °C, 20 min), the sample was dried and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel. (Lanes 1, 17) DNA, dark control, no piperidine treatment; (lanes 2, 16) irradiated DNA without riboflavin; (lanes 3, 15) incubated DNA without drug; (lanes 4, 14) incubated DNA with BPO; (lanes 5, 13) incubated DNA with CoCl2; (lanes 6, 12) incubated DNA with DMS; (lanes 7, 11) irradiated DNA with riboflavin; (lanes 8, 10) incubated DNA in the presence of CoCl2 and BPO; (Lane 9) Maxam–Gilbert sequencing reactions G+A. Lanes 1–8 (10–17) show the result of DNA duplex (quadruplex).

Recently, Hurley et al. has reported that even the cationic porphyrin which has the appropriate physicochemical properties for intercalating between guanine tetrads, including a similar molecular size, a flat porphyrin chromophore, free rotatable substituents, and positive charges could not intercalate into very stable quadruplex DNA structure and that the more favored binding is apparently via stacking at the end of guanine tetrad while leaving the quadruplex DNA network intact. Moreover, there exist K+ ions which coordinate O6 of
guanine bases within the G-tetrad, probably. Thus, it might be difficult for Co(II) or riboflavin to enter between G-tetrad layers. Although it is easy to imagine the cleavage of guanines in the same G-tetrad layer, the cleavage pattern observed in Figure 8 seems unique if quadruplex DNA ODN 4 adopts a probable structure shown in Figure 7b or 7c, because the oxidized four guanines did not exist in the same G-tetrad plane. These cleavage results of quadruplex DNA have never been reported and the results seem to be indicative of an important inherent electronic property of quadruplex DNA. The elucidation of the 5'-G selective cleavage seems to require ab initio calculation of HOMO in quadruplex. Thus, the ab initio calculation of quadruplex is now in progress.

Conclusion

I have examined the oxidation of DNA-RNA hybrid or quadruplex DNA with Co(II) and BPO (experimental HOMO mapping method for B-form DNA duplex). Toward DNA-RNA hybrid, a characteristic cleavage pattern which reflects the conformational change from DNA duplex to DNA-RNA hybrid was observed and the result of experimental HOMO mapping of DNA-RNA hybrid had a very similar tendency to the calculated distribution of HOMOs. Toward quadruplex DNA, the unique cleavage pattern, 5'-G selective cleavage in consecutive guanines, was observed for the first time by using riboflavin or Co(II)-BPO. These results seem to be indicative of an important inherent electronic property of quadruplex DNA. For the elucidation of the 5'-G selective cleavage, ab initio calculation of HOMO in quadruplex is highly desirable.

Experimental Section

General: Riboflavin was purchased from Nacalai Tesque Co., Ltd. Calf thymus DNA (1 g) was purchased from Pharmacia Biotech. Cobalt chloride (CoCl₂) was purchased from Nacalai Tesque Co., Ltd. Benzoyl peroxide (BPO) was purchased from Wako Pure Chemical Industries, Ltd. Calf thymus DNA (1 g) was purchased from Pharmacia Biotech. The oligodeoxynucleotides were purchased from Pharmacia Biotech. T4 polynucleotide kinase was purchased from NIPPON GENE (10 units/μl) and γ-[³²P]-ATP (10 mCi/ml) was from Amersham. All aqueous solutions utilized purified water (MILLIPORE, Mili-Q sp UF). Photoirradiation at 366 nm was carried out using a Cosmo BIO CSF-20AF transilluminator. A GIBCO BRL Model S2 sequencing gel electrophoresis apparatus was used for polyacrylamide gel electrophoresis (PAGE). The gels were analyzed by autoradiography with a Bio-Rad Model GS-700 imaging densitometer and Bio-Rad Molecular Analyst software (Version 2.1).

Preparation of 5'-³²P-End-Labeled ODN

The oligonucleotides (ODNs, 400 pmol strand concentration) were 5'-end-labeled by phosphorylation with 4 μL of [γ-³²P]-ATP and 4 μL of T4 polynucleotide kinase using standard procedures. The 5'-end-labeled oligonucleotides were recovered by ethanol precipitation and further purified by 15% preparative nondenaturing gel electrophoresis and isolated by the crush and soak method.

DNA Cleavage Experiments Using Co(II) and BPO

The ³²P-end-labeled ODNs were hybridized to their complementary ODNs (2.5μM, strand concentration) in 10 mM sodium cacodylate buffer pH 7.0. Hybridization was achieved by heating the samples to 90 °C for 5 min and then slowly cooling the samples to room
temperature. The 32 P-end-labeled duplex solutions were added to a 1.5 mL Eppendorf tube containing 35 μL of MilliQ water, 20 μL of 50 μM aqueous cobalt chloride or 50 μM cobalt acetate solution (15 μM, final conc), and 5 μL of freshly prepared 1 mM benzoyl peroxide (BPO) solution in acetonitrile (50 μM, final conc). After incubation at 37 °C, the solutions were diluted with 10 μL of calf thymus DNA (50 μM, base pair conc) and 10 μL of 3 M sodium acetate buffer (pH 5.2). After precipitation with cold ethanol (800 μL) for 20 min at −80 °C followed by centrifugation (15 min at 0 °C, 1.5 x 10⁴ rpm), the resulting DNA pellets were washed with cold 80% ethanol and dried under vacuum using a Speedvac. The DNA pellets were treated with 1 M piperidine for 20 min at 90 °C, the solution was evaporated under vacuum, and the piperidine was coevaporated with water twice. The radioactivity of the samples was then assayed using an Aloka 1000 liquid scintillation counter and the dried DNA pellets were resuspended in 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). All reactions, along with Maxam-Gilbert G+A sequencing reactions, were heat denatured at 90 °C for 3 min and quickly chilled on ice. The samples (1-2 μL, 2-5 x 10³ cpm) were loaded onto 12 % polyacrylamide/7 M urea sequencing gels and electrophoresed at 1900 V for approximately 2 hand transferred to a cassette and stored at −80 OC with Fuji X-ray film (RX-U). The gels were analyzed by autoradiography with a densitometer and BIORAD Molecular Analyst software (version 2.1). The intensities of the spots resulting from piperidine treatment were determined by volume integration.

Cleavage of 32 P-5’-End-Labeled ODNs by Photoirradiation in the Presence of Riboflavin

32 P-5’-end-labeled ODNs were hybridized to the complementary strand (2.5 μM, strand concentration) in 10 mM sodium cacodylate buffer, pH 7.0. Hybridization was achieved by heating the sample at 90 °C for 5 min and slowly cooling to room temperature. The 32 P-5’-end-labeled ODN duplex (2.0 x 10⁵ cpm ) containing riboflavin was irradiated with a transilluminator at 0 °C for 40 min. After irradiation, all reaction mixtures were ethanol precipitated with the addition of 10 μL of calf thymus DNA (50 μM, base pair conc) and 10 μL of 3 M sodium acetate and 800 μL of ethanol. The precipitated DNA was washed with 100 μL of 80% cold ethanol and dried in vacuo. The operations were carried out as described for the DNA cleavage experiment using Co(II) and BPO.

Theoretical Calculations

All calculations were performed at the HF/6-31G* levels (for HOMO calculation) utilizing GAUSSIAN94 program package. Geometries of stacked methylated nucleobases at N1 (pyrimidine base) and N9 (purine base) were constructed as follows. The corresponding DNA-RNA hybrid 5-mers were built up using the Insight II program (Version 97.0) with standard DNA-RNA hybrid (A-form) helical parameters which have been optimized by X-ray crystallographic analysis of relevant monomers and X-ray diffraction data of polymers. All the sugar backbones of the duplex were removed except for the deoxyribose C1’ carbon and C1’ H and two H atoms were then attached to the C1’ methine to complete stacked N methylated nucleobases, keeping the position of all atoms fixed. HOMOs of the calculated 5-mers were displayed graphically using Gaussian03.

CD Measurement

CD spectra were measured on a JASCO J-720 spectropolarimeter. In DNA-RNA hybrid or DNA duplex, DNA 1 and RNA 3 (DNA 2) (100 μM, base concentration in each DNA or RNA) were hybridized in 10 mM sodium cacodylate buffer at pH 7.0 and 100 mM NaCl. Hybridization was achieved by heating the samples to 90 °C for 5 min and then slowly cooling
the samples to room temperature. The sample solutions were measured at 37 °C. In quadruplex DNA, DNA 4 or 5 (6 µM, strand concentration) was hybridized in 10 mM sodium cacodylate buffer at pH 7.0 and 100 mM KCl. Hybridization was achieved by heating the samples to 90 °C for 10 min and then slowly cooling the samples to room temperature. The sample solutions were measured at 37 °C. CD data were transformed into molar ellipticity [θ] in the units of degree cm²/dm of monomer subunits.

References


Chapter 5

Guanine Selective DNA Alkylation by Naphthalimide and Naphthaldiimide Derivatives Possessing Epoxy Side Chain

Abstract: Altromycin B and kapurimycin A are new members of the pluramycin family antibiotics and alkylate N7 of guanine (G) in duplex DNA at an epoxide subunit attached to the C2 position of the pyranone ring. They are structurally similar to an aglycon part, but an alkenyl epoxide subunit has an opposite absolute configuration. Therefore, it is likely that 5'G or 3'G selective alkylation to the intercalation site of these antibiotics arises from the absolute configuration of epoxide subunit. To clarify this point, I have synthesized naphthalimide (NI) and naphthaldiimide (NDI) possessing an enantiomeric epoxy side chain because NDI is a well known DNA intercalator. Then, the comparative G alkylation experiments were carried out and the difference in binding affinity between NI and NDI was investigated by UV-vis and circular dichroism (CD) spectroscopies. Both enantiomeric NI epoxides, which do not intercalate into DNA so strongly, showed equal guanine cleavage bands. In contrast, highly selective G bands were observed with NDI (S)-epoxide preferentially at 3' G of 5'GG-3' sequence and with NDI (R)-epoxide preferentially at 5' G of 5'GG-3' sequence. These results are interpreted by the strong binding of NDI to DNA by threading intercalation and the absolute configuration of epoxide subunit.
Introduction

Altromycin B (1) and kapurimycin A (2) are new members of the pluramycin family antibiotics and alkylate N7 of guanine (G) in duplex DNA at an epoxide subunit attached to the C2 position of the pyranone ring. The high efficiency of the selective alkylation of the guanine at N7 implies that the epoxide subunit is placed in the major groove of DNA with an appropriate alignment to N7 of guanine through pre-covalent intercalation of the aromatic moiety, as previously suggested for aflatoxin B1 and 1,6,8. The observed 5'-AG*-3' selectivity (* denotes the alkylated site) for the guanine alkylation by 1 was accounted for by a preferential binding to this sequence through a DNA-sugar interaction. Preferential 5' G*G-3' selectivity observed with 2 was accounted for a selective intercalation of 2 into the GG step because of the stabilization of the intercalated complex based on stacking interaction. These antibiotics 1 and 2 are structurally similar to an aglycon part, but an alkenyl epoxide subunit is opposite absolute configuration. Therefore, it is likely that 5'G or 3'G selective alkylation to the intercalation site of 1 or 2 arises from the absolute configuration of epoxide subunit. To clarify this point, comparative G alkylation experiments using an enantiomeric pair of a DNA intercalator have been carried out. It is well known that naphthalimide (NDI) intercalates into DNA. Therefore, I have synthesized NI and NDI derivatives possessing enantiomeric epoxy side chain and examined the comparative G alkylation experiments.

Results and Discussion

Synthesis of Naphthalimide (NI) and Naphthaldiimide (NDI) Derivatives Possessing Epoxy Side Chain

To assess the effect of structures on the sequence selectivity, I have investigated G alkylation by naphthalimide derivatives (NI) 3 and naphthaldiimide derivatives (NDI) 7 bearing an epoxide subunit of known absolute configurations. These compounds were synthesized via a synthetic route shown in Scheme 1 and Scheme 2.
I synthesized naphthalimide derivatives 3 from 1,8-naphthalimide, which was coupled with (2S)-(+) or (2R)-(−)-glycidyl 3-nitrobenzenesulfate as shown in Scheme 1. Next, I synthesized naphthalidimide derivative 7 from 1,4,5,8-naphthalenetetracarboxylic dianhydride as shown in Scheme 2. Treatment of enantiomeric or racemic 1-amino-2,3-propanediol isopropylidene ketal and ethylamine hydrochloride with 1,4,5,8-naphthalenetetracarboxylic dianhydride provided compound 5. After deprotection of 1,2-diol, the stereospecific transformation of 6 gave the targeted chiral naphthalidimide derivative 7.

Analysis of Guanine Alkylation Sequence Selectivity for NI 3 and NDI 7

Sequence selectivities of 3 and 7 were investigated using a 33-mer oligodeoxynucleotide (ODN), 5'-CGTTATCATTGGTTATCATTGGGTTATCATTCG-3', having 5'-'GG-3' and 5'-GGG-3' sequences (underline). Incubation of the double-stranded 32P-5'-end-labeled ODN with 3 and 7 at 37 °C for 60 min, followed by treatment with hot piperidine, resulted in a highly selective G cleavage (Figure 1). As shown in Figure 1, (S)-3 showed equal guanine bands (lane 4). In contrast, highly selective G bands were observed with (S)-7 preferentially at 3' G of 5'-GG-3' sequence (lane 2) and with (R)-7 preferentially at 5' G of 5'-GG-3' sequence (lane 1). Nakatani et al. have reported that highly 5' G selective alkylation of the GG sequence by kapurimycin A may arises from a selective intercalation into the GG step and that stacking interaction with both 5' and 3' Gs is a basis for the stabilization of the intercalated complex. This difference in sequence selectivity between NI 3 and NDI 7 may arise from the difference in binding affinity, that is, NDI interacts to DNA more strongly than NI. Then, I investigated the difference in binding affinity between NI and NDI.

Characterization of DNA/NI and DNA/NDI Complexes

Binding of the NI or NDI to herring sperm DNA was investigated by UV-vis and circular dichroism (CD) spectroscopies. NI derivative 4 and racemic NDI derivative rac-5 were used for the analysis of binding affinity. NI derivative 4 was easily synthesized from 1,8-naphthalic anhydride which was coupled with allylamine. At first, equilibrium binding constants for NDI rac-5 and NI 4 were measured by titrating a buffered solution (10 mM sodium cacodylate, pH=7.0) of these drugs with herring sperm DNA and monitoring the decrease in absorbance of the sample. The absorption band for rac-5 showed a pronounced hypochromic effect in the presence of herring sperm DNA, but the absorption band of NI 4...
showed almost no change upon addition of herring sperm DNA (Figure 2). The binding curve of rac-5 ($r < 0.2$) was well fitted with the non-cooperative site-exclusion model of McGhee and von Hippel. The apparent neighbor $n$ and the resulting binding constants $K$ of rac-5 were 4.5 and $1.1 \times 10^6 \text{M}^{-1}$, respectively. This result was consistent with the previous studies, in which binding constants of NDI derivatives ranged from $1.1 \times 10^5 \text{M}^{-1}$ to $5.0 \times 10^5 \text{M}^{-1}$ with $n$ being $2^{10,20}$ and ca. 5.0.$^{15}$

![Figure 2. UV-vis titration of NDI rac-5 (a) and NI 4 (b) with herring sperm DNA in 10 mM sodium cacodylate buffer (pH 7.0). Drug concentration was 50 $\mu$M. DNA solution (herring sperm DNA (1.5 mM base concentration) and 50 $\mu$M drug in 10 mM sodium cacodylate buffer (pH 7.0)) was added.](image)

Information about the geometry of the DNA/NI or DNA/NDI derivatives intercalated complexes can be obtained from CD spectroscopy. Binding of an achiral molecule within a chiral environment, such as that afforded by right-handed double-helical DNA, can lead to an induced optical activity for the bound species (ligand). This is manifested in the appearance of CD absorption band, assignable to the ligand but observed only in the presence of DNA. Provided the ligand absorbs light in a region where the DNA is transparent, it is straightforward to assign the new band to the ligand. This is well known effect observed for a variety of DNA intercalators. In this case, only NDI rac-5 exhibited an induced CD (Figure 3). The addition of rac-5 to a DNA solution led to the decrease of the characteristic Cotton effects ascribed to B-form DNA, the positive CD at 275 nm and the negative CD at 245 nm, together with the increase of the negative induced CD at 420 nm due to the formation of NDI rac-5–DNA complex. This result shows the structural change of DNA induced by intercalation of rac-5 to DNA duplex.

![Figure 3. CD spectral change due to the interaction of rac-5 with herring sperm DNA. (Dotted line) a solution of herring sperm DNA (100 $\mu$M) in the absence of drug; (dashed line) a solution of herring sperm DNA (100 $\mu$M) in the presence of 4; (solid line) a solution of herring sperm DNA (100 $\mu$M) in the presence of rac-5 in 10 mM sodium cacodylate buffer (pH 7.0) at 20 °C.](image)

**Mechanism of the Absolute Configuration of Epoxide Subunit Selective Guanine**

Previously, Wilson et al. have reported that a symmetric naphthalimide with alkyl amino substituents both associated and dissociated from DNA more slowly than classical intercalators with similar binding constants from stopped-flow kinetics experiments and that these results supported a threading intercalation model, with one charged diimide substituent in each of the DNA grooves rather than with both side chains in the same groove, for the diimide complex with DNA. They have also indicated that the naphthalimide bound to poly[d(G-C)] 20–25 times strongly than to poly [d(A-T)], from spectrophotometric binding studies. After that, Rill et al. have reported that alkylamine substituted naphthalimide and naphthalimide preferred binding to G:C base pairs and that the binding constant of the diimide was approximately one order of magnitude higher than those of the monoimide. Therefore, the guanine selective cleavage of both NI and NDI may arise from preferential
binding to G:C base pairs in DNA because of the stabilization of intercalated complex via strong stacking with both 5’ and 3’ side Gs.\(^\text{13}\)

It is likely that the difference in cleavage pattern between NI and NDI is derived from the binding ability of these molecules. Since NDI binds to DNA by the threading mechanism and associates and dissociates from DNA much more slowly than normal intercalator such as ethidium,\(^\text{14}\) its cleavage selectivity seems to depend on the absolute configuration of the epoxide subunit. Whereas the orientation of (S)-7 epoxide is highly suitable for back side attack of guanine N7 of 3’ G of 5’-GG-3’ site, a similar intercalation of (R)-7 epoxide places the epoxide ring inaccessible for \(\text{S}_\text{N}2\) attack, as shown in Figure 4a. Therefore, NDI (S)-7 showed the 3’ G selective cleavage of 5’-GG-3’ step. The 5’ G selective cleavage of NDI (R)-7 can be explained in the same way (Figure 4b). On the other hand, optical active NI derivatives (S)-3 and (R)-3 associate and dissociate so rapidly that the absolute configuration of epoxide subunit has little effect for the guanine alkylation. In fact, Wilson \textit{et al.} has reported that naphthalimide derivatives associate and dissociate so rapidly that their kinetics are outside of the stopped-flow range.\(^\text{14}\)

**Conclusion**

I have synthesized NI and NDI derivatives possessing enantiomeric epoxy side chain and examined the comparative G alkylation experiments. Consequently, only NDI epoxide showed the preferential alkylation of 5’-or 3’-G of 5’-GG-3’ sequence. These results are explained by the strong binding of NDI to DNA by threading intercalation and the absolute configuration of epoxide subunit. Substituted 1,8-NIs and bis NIs\(^\text{24}\) (Figure 5) have been shown to demonstrate dramatic anticancer activity\(^\text{25,26}\) and certain members of both series have already entered into clinical trials.\(^\text{27}\) Therefore, these enantiomeric NI and NDI epoxide derivatives might be the candidates for anticancer or antiviral agents.

![Figure 4. Binding model for DNA-NDI 7 complex. (a) (S)-7-GG/CC complex; (b) (R)-7-GG/CC complex. These models were obtained from optimization of 7-5’-d(TGGT)-3’/5’-d(ACCA)-3’ complex using Amber* force field in water by means of Macromodel. After optimization, AT base pairs were removed. Guanine N7 in DNA and epoxide oxygen of NDI were shown in dark gray. DNA and 7 were shown in balck, and light gray, respectively.](image)

![Figure 5. Substituted 1,8-naphthalimides and bis naphthalimides with anticancer activity.](image)
Experimental Section

General: $^1$H NMR spectra were measured with Varian Mercury 400 (400 MHz) spectrometer. $^13$C NMR spectra were measured with JEOL JNM α-500 (125 MHz) spectrometer. Coupling constants ($J$ values) are represented in hertz. The chemical shifts are expressed in ppm downfield from tetramethylsilane, using residual chloroform ($\delta = 7.24$ in $^1$H NMR, $\delta = 77.0$ in $^13$C NMR) and dimethylsulfoxide ($\delta = 8$ in $^1$H NMR, $\delta = 39.5$ in $^13$C NMR) as an internal standard. Melting point were obtained on a Yanaco micro melting point apparatus. EI mass spectra and FAB Mass spectra were recorded on a HX-110 and JEOL JMS HX-110 spectrometer, respectively. Wakogel C-200 was used for silica gel flash chromatography. Precoated TLC plates Merck silica gel 60 F 254 was used for monitoring reactions. Herring sperm DNA (5 × 10$^3$ mg/L) was purchased from GIBCO BRL, LIFE TECHNOLOGIES. The oligodeoxynucleotides were purchased from Amersham Pharmacia Biotech. T4 polynucleotide kinase was purchased from NIPPON GENE (10 units/µL) and γ-$^32$P-ATP (10 mCi/ml) was from Amersham. All aqueous solutions utilized purified water (MILLIPORE, Mili-Q sp UF). A GIBCO BRL Model S2 sequencing gel electrophoresis apparatus was used for polyacrylamide gel electrophoresis (PAGE).

$N$-[(2S)-Oxiranylmethyl]-1,8-naphthalimide (3)

To a suspension of sodium hydride (60%, 13.4 mg, 0.56 mmol) in $N,N$-dimethylformamide (5 mL) was added a solution of 1,8-naphthalimide (100 mg, 0.51 mmol) in $N,N$-dimethylformamide (2 mL) at 0 °C, and the mixture was stirred at 65 °C for 1 h. After cooling down to ambient temperature, to this mixture was added a solution of (2S)-(+) glycidyl 3-nitrobenzenesulfonate (263 mg, 1.01 mmol) in $N,N$-dimethylformamide (2 mL) at ambient temperature, and the mixture was stirred at 60 °C for 1 h. After cooling down to ambient temperature, the mixture was diluted with sat. aq. ammonium chloride and extracted with chloroform (50 mL × 3). The combined organic layers were dried with magnesium sulfate, filtered and evaporated. The crude product was purified by column chromatography on silica gel, eluting with 10% methanol in chloroform to give 3 (92.4 mg, 72%) as a white solid: mp 169 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.62 (d, 2H, $J = 8.0$ Hz), 8.22 (d, 2H, $J = 8.4$ Hz), 7.76 (t, 2H, $J = 8.0$ Hz), 4.52 (dd, 1H, $J = 4.8$, 5.2 Hz), 4.28 (dd, 1H, $J = 5.2$, 5.6 Hz), 3.35 (m, 1H), 2.77-2.81 (2H); $^13$C NMR (125 MHz, CDCl$_3$) $\delta$ 164.26, 134.23, 131.65, 131.53, 128.26, 126.99, 122.41, 49.31, 46.57, 41.78; MS (EI) m/z (%) 253 (M$^+$), 222 (100), 210 (64), 180 (61), 152 (49), 126 (51); HRMS (EI) calcd for C$_{15}$H$_{11}$O$_3$N (M$^+$) 253.0738, found 253.0735.

$N$-(1-propenyl)-1,8-naphthalimide (4)

To a suspension of 1,8-naphthalic anhydride (1005 mg, 5.07 mmol) in toluene (20 mL) was added allyl amine (434 mg, 7.61 mmol), and the mixture was refluxed for 5.5 h. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography on silica gel, eluting with 10% ethyl acetate in toluene to give 4 (1055 mg, 88%) as a light orange solid: mp 137 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.62 (d, 2H, $J = 7.3$ Hz), 8.22 (d, 2H, $J = 8.4$ Hz), 7.76 (t, 2H, $J = 8.4$ Hz), 6.00 (ddt, 1H, $J = 17.2$, 10.3, 5.7 Hz), 5.33 (dd, 1H, $J = 17.0$, 1.3 Hz), 4.80 (dt, 2H, $J = 5.7$, 1.3 Hz); $^13$C NMR (125 MHz, CDCl$_3$) $\delta$ 163.94, 134.02, 132.18, 131.63, 131.35, 128.22, 126.95, 122.62, 117.57, 42.41; MS (FAB) m/z (%) 238 [(M+H)$^+$]; HRMS (FAB) calcd for C$_{15}$H$_{12}$O$_2$N [(M+H)$^+$] 238.0790, found 238.0860.
N-[3,3-Dimethyl(2,4-dioxolanyl)methyl]-N' -ethyl-1,4,5,8-naphthalimide (5)

To a suspension of ethylamine hydrochloride (451 mg, 5.53 mmol) in tetrahydrofuran (35 mL) was added triethylamine (0.80 mL, 5.70 mmol) at ambient temperature, and the mixture was stirred for 30 min. To this mixture was added 1-amino-2,3-propanediol isopropylidene ketal (552 mg, 4.21 mmol) and 1,4,5,8-naphthalenetetracarboxylic dianhydride (753 mg, 2.81 mmol) at ambient temperature and the mixture was refluxed for 6 h. The resulting mixture was filtered and the filtrate was concentrated in vacuo. The crude product was purified by column chromatography on silica gel, eluting with 5% methanol in chloroform to give 5 (459 mg, 40%) as a yellow solid: mp 201 °C; 1H NMR (400 MHz, CDCl3) δ 8.75 (s, 4H), 4.60 (dd, 1H, J = 7.4, 12.4 Hz), 4.56 (dddd, 1H, J = 4.0, 5.2, 6.0, 7.4 Hz), 4.25 (q, 2H, J = 7.1 Hz), 4.15 (dd, 1H, J = 4.0, 12.4 Hz), 4.12 (dd, 1H, J = 6.0, 8.8 Hz), 3.90 (dd, 1H, J = 5.2, 8.8 Hz), 1.47 (s, 3H), 1.34 (t, 3H, J = 7.1 Hz), 1.47 (s, 3H); 13C NMR (125 MHz, CDCl3) δ 162.97, 162.56, 131.26, 130.92, 126.99, 126.67, 126.45, 109.85, 73.32, 67.70, 43.46, 36.07, 26.69, 25.50, 13.29; MS (FAB) m/e (%) 369 [(M+H)+]; HRMS (FAB) calcd for C19H17O6N2 [(M+H)+] 369.1085, found 369.1099.

N-(2-Oxiranylmethyl)-N' -ethyl-1,4,5,8-naphthalimide (7)

To a suspension of 6 (20.0 mg, 54.3 mmol) and pyridinium p-toluene sulfonate (0.2 mg, 0.80 μmol) in dichloromethane (5 mL) was added trimethyl orthoacetate (20 μL, 0.16 mmol) at ambient temperature, and the mixture was stirred for 1 h. The volatiles were evaporated and residual methanol was removed in vacuo. To the resulting materials were added dichloromethane (5 mL) and acetyl bromide (20 μL, 0.27 mmol) at ambient temperature and the mixture was stirred at ambient temperature for 1 h. The volatiles were evaporated. To the resulting materials were added methanol (10 mL) and potassium carbonate (113 mg, 0.82 mmol) at ambient temperature and the mixture was stirred vigorously at ambient temperature for 2 h. The mixture was poured into saturated aqueous ammonium chloride (20 mL) and extracted with chloroform (50 mL x 3). The combined organic layers were dried with magnesium sulfate, filtered, and evaporated. The crude product was purified by column chromatography on silica gel, eluting with 10% methanol in chloroform to give 7 (12.8 mg, 68%) as a pale red solid: mp 232 °C; 1H NMR (400 MHz, DMSO-d6) δ 8.64 (s, 4H), 4.84 (d, 1H, J = 5.2 Hz), 4.63 (t, 1H, J = 5.8 Hz), 4.23 (dd, 1H, J = 8.4, 12.4 Hz), 4.09 (q, 2H, J = 7.1 Hz), 4.00 (dd, 1H, J = 4.8, 12.4 Hz), 3.91 (m, 1H), 3.37-3.46 (2H), 1.23 (t, 3H, J = 7.1 Hz); 13C NMR (125 MHz, DMSO-d6) δ 162.78, 162.34, 130.29, 126.34, 126.17, 126.04, 126.00, 68.28, 43.69, 35.20, 12.94; MS (FAB) m/e (%) 351 [(M+H)+]; HRMS (FAB) calcd for C16H19O6N2 [(M+H)+] 351.0980, found 351.0983.
Preparation of 5'-32P-End-Labeled ODN

The oligonucleotides (ODNs, 400 pmol-strand) were 5'-end-labeled by phosphorylation with 4 μL of [γ-32P]ATP and 4 μL of T4 polynucleotide kinase using standard procedures. The 5'-end labeled oligonucleotides were recovered by ethanol precipitation and further purified by 15% preparative nondenaturing gel electrophoresis and isolated by the crush and soak method.

DNA Cleavage Experiments

The 32P-end-labeled ODNs were hybridized to their complementary ODNs (2.5 μM, strand concentration) in 10 mM sodium cacodylate buffer (pH 7.0). Hybridization was achieved by heating the samples to 90 °C for 5 min and then slowly cooling the samples to room temperature. The 32P-end-labeled duplex solutions (40 μL) were added to a 1.5 mL Eppendorf tube containing 55 μL of MilliQ water, 5 μL of 1 mM drug in acetonitrile (50 μM, final conc). After incubation at 37 °C for 60 min, the solutions were diluted with 10 μL of calf thymus DNA (50 μM, base pair conc) and 10 μL of 3 M sodium acetate buffer (pH 5.2). After precipitation with cold ethanol (800 μL) for 20 min at −80 °C followed by centrifugation (15 min at 0 °C, 1.5 x 10^4 rpm), the resulting DNA pellets were washed with cold 80% ethanol and dried under vacuum using a Speedvac. After the DNA pellets were treated with 1 M piperidine for 20 min at 90 °C, the solution was evaporated under vacuum, and the piperidine was coevaporated with water twice. The radioactivity of the samples was then assayed using an Aloka 1000 liquid scintillation counter, and the dried samples were dissolved in 80% formamide loading buffer (to reach to a radiation density of 2000-3000 cpm/μL) and electrophoresed through a denaturing 12% polyacrylamide/7 M urea gel (1900 V, 1.5 h). The gels were exposed to X-ray film with an intensifying screen at −70 °C.

Determination of Binding Constants of NDI rac-5 to Herring Sperm DNA

A buffered solution (10 mM sodium cacodylate buffer at pH 7.0) of NDI rac-5 (50 μM in a 1.0 cm cuvette) was titrated with a mixture solution of NDI rac-5 (50 μM) and herring sperm DNA (1.5 mM, base concentration). The optical density of the solution at 382.6 nm was measured initially and after each addition on a JASCO V-550 UV/VIS spectrophotometer. Scatchard plot for for the binding of NDI rac-5 to DNA (r < 0.2) was fit by nonlinear least square analysis to the McGhee and von Hippel equation governing random noncooperative binding to a lattice.

CD Measurement

CD spectra were measured on a JASCO J-720 spectropolarimeter. Solutions were prepared containing drug (50 μM), herring sperm DNA (100 μM, base concentration) and 10 mM sodium cacodylate buffer at pH 7.0. The sample solution was measured at 20 °C. CD data were transformed into molar ellipticity [θ] in the units of degree cm^2/dm of monomer subunits.
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List of Publications

Chapter 1 Mapping of the Hot Spots for DNA Damage by One-Electron Oxidation: Efficacy of GG Doublets and GGG Triplets as a Trap in Long-Range Hole Migration.

Chapter 2 Experimental and Theoretical Studies on the Selectivity of GGG Triplets toward One-Electron Oxidation in B-Form DNA.

-GG- Specific Cleavage via Hole Hopping through Double-Stranded DNA.

Chapter 3 Mapping of Highest Occupied Molecular Orbitals of Duplex DNA by Cobalt-Mediated Guanine Oxidation.
DNA HOMO as a New Landmark for Nucleic Acid Properties. Ab Initio Calculations and Experimental Mapping.

**Chapter 4**
Mapping of the Hot Spots for DNA-RNA Hybrid and Quadruplex DNA by Cobalt-Benzoyl Peroxide Oxidation.
Saito, I.; Nakamura, T.; Okamoto, A.; Nakatani, K. to be submitted.

**Chapter 5**
Guanine Selective DNA Alkylation by Naphthalimide and Naphthaldimide Derivatives Possessing Epoxy Side Chain.

**Other Publications**


4. Design of photochemical DNA-cleaving molecules via electron transfer.


List of Presentations


2. “Guanine-Guanine Specific Cleavage in Duplex DNA by Photo-Electron Transfer”
   70th Annual Meeting of Chemical Society of Japan, Tokyo, Japan, March, 1996.

3. “Sequence- and Conformation-Dependent Photoinduced-Electron Transfer from DNA”

4. “Sequence- and Conformation-Dependent Photo-Induced Electron Transfer from DNA”
   76th Annual Meeting of Chemical Society of Japan, Yokohama, Japan, March, 1999.

5. “Guanine-Guanine Stacking Rule for Specific Cleavage of Duplex DNA by Photoinduced Electron Transfer”

6. “GG- Specific Cleavage via Hole Hopping through Double-Stranded DNA”
   T. Nakamura, C. Dohno, K. Nakatani, I. Saito.

7. “Mapping Hot Spots for One-Electron Oxidation in Double Stranded DNA”

8. “HOMO Mapping of B-DNA Duplex by Using Metal Ion and Oxidizing Agent”
   76th Annual Meeting of Chemical Society of Japan, Yokohama, Japan, March, 1999.

9. “DNA HOMO as a New Landmark for Nucleic Acid Properties”