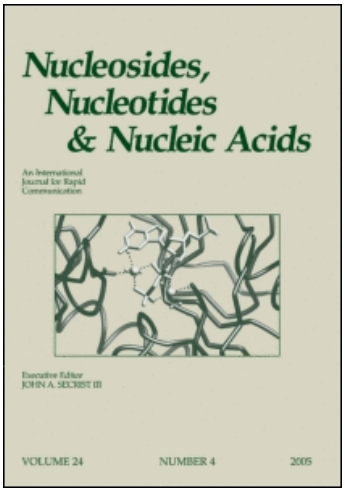

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[a] Institute of Advanced Energy, Kyoto University, Gokasho, Uji, Kyoto, Japan [b] CREST, JST, Gokasho, Uji, Kyoto, Japan [c] Kyoto Nanotechnology Cluster Project, Gokasho, Uji, Kyoto, Japan

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BIOPHYSICAL STABILITY AND ENZYMATIC RECOGNITION OF OXANINE IN DNA

Seung Pil Pack, Akihiro Doi, Mitsuru Nonogawa, Nagendra K. Kamisetty, Kamakshaiah C. Devarayapalli, Tsutomu Kodaki, and Keisuke Makino

□ *Institute of Advanced Energy, Kyoto University, Gokasho, Uji, Kyoto, Japan, CREST, JST, Gokasho, Uji, Kyoto, Japan, and Kyoto Nanotechnology Cluster Project, Gokasho, Uji, Kyoto, Japan*

□ *Oxanine (Oxa), which is one of the major products generated from guanine by nitrosative oxidation and is as long-lived as Gua in DNA, has been thought to be one of the major causes for NO-induced DNA damage. In the present study, using several synthetic Oxa-containing oligodeoxynucleotides, biophysical stability and enzymatic recognition of Oxa was investigated in DNA strands. It was found that Oxa did not mediate marked distortion in the whole DNA structure although Oxa pairing with 4 normal bases decreased thermal stability of the DNA duplexes compared to Gua:Cyt base pair. Regarding the responses of the DNA-relevant enzymes to Oxa, it was determined that Oxa was recognized as Gua except that DNA polymerases incorporated Thy as well as Cyt opposite Oxa. These results imply that Oxa tends to behave as a kind of naturally occurring base, Gua and therefore, would be involved in the genotoxic and cytotoxic threats of NO in cellular system.*

Keywords Oxanine; oligodeoxynucleotide; thermal stability; DNA-relevant enzymes

INTRODUCTION

In 1996, it was demonstrated that oxanine (Oxa, O) is generated as one of the major lesions in NO-induced oxidative threats and that Oxa is formed together with xanthine (Xan), the major product, with the molar ratio of 1:3 when 2′-deoxyguanosine (dGuo) or DNA is treated with NO or in weakly acidic HNO_2 as shown in Figure 1.[1] The whole reaction mechanism for the Oxa formation also was reported: Gua-diazoate is an intermediate produced after the nitroso-Gua intermediate formation is converted to the diazonium

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Address correspondence to Keisuke Makino, Institute of Advanced Energy, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan. E-mail: kmak@iae.kyoto-u.ac.jp

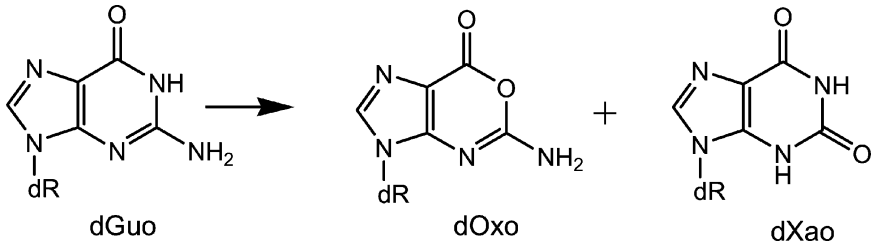

FIGURE 1 Formation of 2′-deoxyoxanosine (dOxo, deoxynucleoside of Oxa) and 2′-deoxyxanthosine (dXao, deoxynucleoside of Xan) from dGuo by NO- or HNO_2- induced nitrosative oxidation.

ion, and then Oxa and Xan are formed through the ring-opening upon release of N_2.[2]

Chemical and biochemical properties of Oxa also have been explored intensively to elucidate the biological implication of Oxa production. It was reported that Oxa is incorporated opposite cytosine (Cyt, C) and thymine (Thy, T) in the DNA polymerase chain elongation.[3] It was also found that Oxa produced in DNA has long half-life due to the stable *N*-glycosidic bond between Oxa and the sugar moiety in its nucleoside, 2′-deoxyoxanosine (dO × o).[4] Recently, it was demonstrated that Oxa reacts with nucleophilic moieties of biomolecules and induces DNA-protein cross-link (DPC) formation.[5]

Oxa-induced DPC formation would be relevant to genotoxic and cytotoxic events caused by NO or HNO_2 in the cellular system and therefore, allow us to explore Oxa repairing system in cell. It has been determined that general BER (base excision repair) system is not effective for repairing Oxa in DNA strands.[6] More plausible Oxa-repairing mechanism would be the nucleotide excision repair (NER) system because such system is found to excise large lesion such as Oxa-mediated DPC.[6] Recently, we have developed a chemical synthesis procedure for preparing Oxa-containing oligodeoxynucleotide (Oxa-ODN),[7] which can be used as probe molecules for identifying the Oxa-related genotoxic events in cellular system.

In this report, we designed several kinds of Oxa-ODNs and analyzed their biophysical stability and recognition by DNA-relevant enzymes to investigate those unsolved problems.

RESULTS AND DISCUSSION

Our previous report revealed that Oxa exists for a long time in DNA strands due to the stable *N*-glycosidic bond,[4] indicating that this lesion could be serious, and therefore, it is essential to analyze its basic biochemical and biophysical nature in detail. In present study, melting temperature (T_m) and CD analyses were carried out by using the synthetic oligodeoxynucleotides (ODN) such as 5′-d(**GXC** TCTCTTCCCTACGAT)-3′ (end-GXC-)

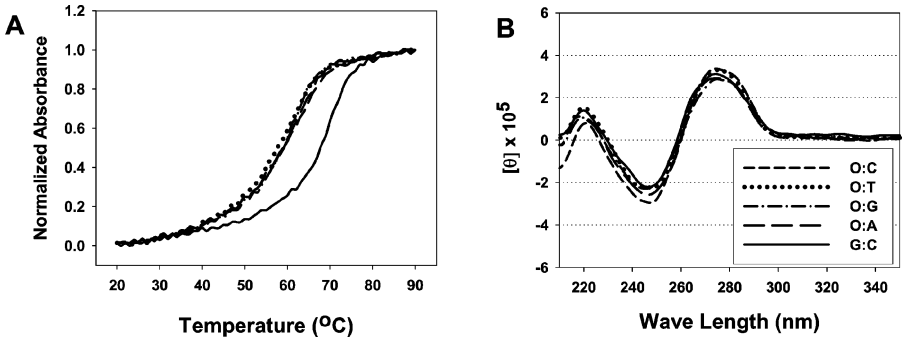

FIGURE 2 Melting temperature (T_m) analysis (**A**) and CD analysis (**B**) of DNA duplexes of mid-GXC- and its complementary (DNA concentration; 4 μM for T_m analysis and 16 μM for CD analysis. Symbols; O:C (mid-dash line), O:T (dot line), O:G (dash-dot line), O:A (long-dash line), and G:C (solid line).

and 5′-d(TCTCTTCC **GXC** CTACGAT)-3′ (mid-GXC-) (**X** = O or G), and their complementary sequences 5′-d(ATCGTAGGGAAGAAG **GNC**)-3′ and 5′-d(TCGTAG **GNC** GGAAGAAG)-3′ (**N** = A, C, T or G). T_m analyses revealed that all the DNA duplexes containing O:N base pairs were less stable than the perfect-matched DNA duplex by 5.04 to 7.27°C in the case of end-GOC- and 6.64 to 9.80°C in the case of mid-GOC (Figure 2A). The order of DNA duplex stabilities indicated by the T_m values was O:C (64.47°C) > O:T (62.45°C) > O:G (62.37°C) > O:A (62.24°C) in the case of end-GOC- and O:C (64.51°C) > O:A (62.05°C) > O:G (61.94°C) > O:T (61.35°C) in the case of mid-GOC-. To investigate whether such weak base-pairings of Oxa influence on the whole DNA structure, CD analysis was performed. CD spectra revealed that single replacement of Oxa in the DNA duplex does not cause marked distortion in the whole DNA structure irrespective of base-paring types of O:N (Figure 2B). However, considering the observed T_m decrease results, multiple replacement of Oxa in DNA duplex might influence the whole DNA structure.

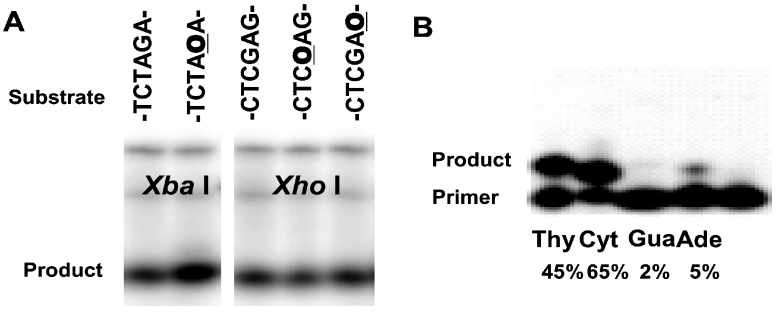

FIGURE 3 Recognition analysis of Oxa in DNA by restriction endonuclease enzymes (**A**) and incorporation efficiencies of normal bases opposite Oxa in the chain elongation using *E. coli* DNA polymerase I Kf (**B**).

Next, the influence of Oxa on the recognition of DNA-relevant enzymes was investigated. The following synthetic ODNs were employed as substrates; 5′-d(**X**CCATT**X**CCTGA**X**TTCTA**X**AGTG)-3′ (**X** = O or G) (23 mer) for nuclease S1 and exonuclease I, and 5′-d(GAGTGCGGC TCTA**X**A [or CTC**X**AG or CTCGA**X**] GCGGCTCAG)-3′ (**X** = O or G) (24 mer) and its complementary DNA strands for *Xba* I and *Xho* I restriction enzymes. When Oxa-ODNs were testified as substrates for nuclease S1 and exonuclease I, the enzymatic functions were almost unchanged by Oxa in DNA. As shown in Figure 3A, it was determined also that restriction endonucleases *Xba* I and *Xho* I recognized and cleaved their specific base-sequences, in which Gua was substituted by Oxa. These results of the enzymatic reactions indicate that Oxa could be considered as Gua in DNA strands by DNA-relevant enzymes. On the other hand, in case of DNA polymerase chain elongation, Oxa in template DNA strand induces Thy incorporation as well as Cyt incorporation. As presented in Figure 3B, the incorporation efficiencies of normal base opposite Oxa were 65% for Cyt, 45% for Thy, and below 5% for Gua and Ade. It might be related to the steric reasons that incorporation of the pyrimidine nucleosides opposite to Oxa is preferred over that of the purine nucleosides.

CONCLUSION

In the present study, using several synthetic Oxa-ODNs, biophysical stability and enzymatic recognition of Oxa in DNA were explored. It was found that Oxa in the DNA duplex does not cause marked distortion in the whole DNA structure. In addition, Oxa in DNA does not make distinctive influence on DNA-relevant enzyme functions. However, Oxa induces the incorporation of Thy as well as Cyt during the DNA polymerase chain elongation. Consequently, Oxa, generated from Gua by NO-induced oxidation, would not be distinguished as abnormal base in DNA but it could mediate GC to AT transversion, which is one of mutagenic problems in cellular system.

EXPERIMENTAL PROCEDURE

Phosphoramidite DNA monomer of Oxa and Oxa-ODNs were prepared according to the chemical synthesis procedure as reported previously.[7] For melting temperature analysis, all the DNA solutions were prepared in a phosphate buffer composed of 1 M NaCl, 10 mM Na_2HPO_4, and 1 mM Na_2EDTA adjusted to pH 7.0. Melting curves of DNA duplexes were obtained for the solutions (4 μM) containing a 1:1 strand ratio of a set of ODNs with an increase in temperature from 20 to 90°C at a rate of 0.2°C/minute. For analysis of the whole DNA structure, CD spectra of DNA duplex were measured from 350 to 210 nm at 25°C with a scan speed 100 nm/minute with a path length of 10 mm. The total strand concentration of the samples

was 16 μM in the same buffer solutions as used for the melting temperature studies.

For the hot-labeling of ODNs, ODNs (800 nM) were incubated with T4 PNK (40 unit) and [γ-^{32}P]ATP (4.5 MBq) in 50 μl of reaction solution at 37°C for 30 minutes. The reaction was terminated by heat deactivation at 75°C for 10 minutes and the hot-labeled ODN solution was separated by using the purification column of CENTRI-SEP of Princeton Separations (Adelphia, NJ, USA). The obtained ODNs were used as substrates for further enzymatic reactions; (a) for nuclease reaction, single DNA strand (100 nM) was treated by nuclease S1 (1 unit) or exonuclease I (1 unit) in 50 μl of reaction solution at 37°C for 30 minutes, (b) for restriction endonuclease reaction, DNA duplexes (100 nM each) were treated by *Xba* I or *Xho* I enzyme (4 unit) in 50 μl of reaction solution at 37°C for 30–60 minutes, (c) for DNA polymerase chain elongation, the template/primer (10 nM each) was incubated with *E. coli* DNA Pol I Kf (0.001 unit) and 4 dNTPs (100 μM each) in 10 μl of reaction solution at 37°C for 3–60 minutes. The reactions were terminated by adding gel loading buffer. The products were separated by 20% denaturing PAGE containing 6 M Urea. The results were analyzed on a phosphorimaging scanner, STORM 820 of GE-Healthcare (Piscataway, NJ, USA).

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