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Biomolecular response of oxanine in DNA strands to T4 polynucleotide kinase, T4 DNA ligase, and restriction enzymes

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ABSTRACT

Oxanine (Oxa), generated from guanine (Gua) by NO- or HNO_2-induced nitrosative oxidation, has been thought to cause mutagenic problems in cellular systems. In this study, the response of Oxa to different enzymatic functions was explored to understand how similarly it can participate in biomolecular reactions compared to the natural base, Gua. The phosphorylation efficiency of the T4 polynucleotide kinase was highest when Oxa was located on the 5′-end of single stranded DNAs compared to when other nucleobases were in this position. The order of phosphorylation efficiency was as follows; Oxa > Gua > adenine (Ade) ~ thymine (Thy) > cytosine (Cyt). Base-pairing of Oxa and Cyt (Oxa:Cyt) between the ligation fragment and template was found to influence the ligation performance of the T4 DNA ligase to a lesser degree compared to Gua:Cyt. In addition, EcoRI and BglII showed higher cleavage activities on DNA substrates containing Oxa:Cyt than those containing Gua:Cyt, while BamHI, HindIII and EcoRV showed lower cleavage activity; however, this decrease in activity was relatively small.

Introduction

In 1996, we reported that oxanine (Oxa, O) could form together with xanthine (Xan) at a molar ratio of 1:3 when 2′-deoxyguanosine or DNA was treated with nitric oxide (NO) or weakly acidic nitrous acid (HNO_2) [1]. This result suggested that Oxa, a deaminated product of guanine (Gua, G) in which the endocyclic amine was oxidized, could be one of the major nucleobase lesions generated from NO-induced oxidative stress in cellular systems. Since then, the chemical and biochemical properties of Oxa have been extensively analyzed to better understand the biological implications of Oxa [2–10]. Oxa has been known to exist for a long time due to the stable *N*-glycosidic bond between the base and sugar moieties of its nucleoside, 2′-deoxyoxanosine (dOxo) [3]. In DNA polymerase chain elongation, Oxa could mediate incorporation of thymine (Thy, T) as well as incorporation of cytosine (Cyt, C); thereby leading to genotoxic GC-to-AT transversion [4,10]. It was also shown that Oxa could induce DNA–protein cross-link (DPC) formation with nucleophilic moieties of biomolecules [8]. These results indicated that, once Oxa was produced in a DNA sequence, Oxa or its DPC formation could increase genotoxic and cytotoxic problems in cellular systems [9].

Recently, we developed a chemical synthesis procedure for preparing synthetic Oxa-containing oligodeoxynucleotides (Oxa–ODNs) and investigated the biophysical properties of Oxa in DNA strands [11]. CD analysis suggested that the presence of Oxa in DNA strands might not considerably distort the whole DNA duplex structure [3,11]. However, in the melting temperature (T_m) analysis, the DNA duplex containing Oxa base-pairings showed lower thermal stability than the perfectly-matched DNA duplex and possessed similar stability as those of other mis-matches such as A:C and G:T. In addition to the biophysical analyses of Oxa in DNA strands, it is necessary to determine the biomolecular response of Oxa to DNA-relevant enzymes or proteins, which would provide important biophysical and biochemical clues to better understanding the *in vivo* behavior of Oxa and its genotoxicity in cellular systems. Moreover, base excision repair (BER) enzymes were found not to effectively recognize Oxa in DNA strands [7,9]; thus, it is highly likely that Oxa, once present in DNA, will participate in DNA related biomolecular interactions.

Therefore, in this study, we prepared several types of synthetic Oxa–ODNs, in which Oxa was located at the ends or middle of the ODNs, and these ODNs were used as substrates for several DNA-binding/recognizing enzymes. The response of Oxa–ODNs to T4 polynucleotide kinase (T4 PNK), T4 DNA ligase, and restriction endonuclease enzymes (BamHI, BglII, EcoRI, EcoRV, and HindIII) were explored to investigate how similarly Oxa can participate in biomolecular interactions compared to the natural base, Gua.

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Materials and methods

Reagents and enzymes. The reagents for oligodeoxynucleotide synthesis (including CPG column and appropriately protected normal nucleosides) were obtained from Glen Researches Co. (Sterling, VA). [γ-^{32}P]ATP was purchased from GE Healthcare (Piscataway, NJ). T4 polynucleotide kinase (T4 PNK) and T4 DNA ligase were acquired from Takara (Shiga, Japan) and Antarctic phosphatase from New England Biotech (Ipswich, MA). Other chemicals reagents were purchased from Wako (Osaka, Japan) and solvents from Nacalai Tesques (Osaka, Japan).

Instrument system. Solid-phase chemical synthesis of DNA oligomers was carried out with an Applied Biosystems 3400 DNA synthesizer [Applied Biosystems (Foster, LA)]. UV spectra of DNA oligomers were measured on a Shimadzu UV-260 UV–vis spectrophotometer equipped with an SPR-5 temperature controller. An RP-HPLC system constructed with a Tosoh PX-8020 (controller), a DP-8020 (pump), a CO-8020 (temperature controller) and a PD-8020 (diode detector) with an Ultron VX-ODS column [150 × 4.6 mm (for analysis) or 250 × 10 mm (for purification), 5 μm; Shinwa Co.] was used. The PAGE results of hot-labeled DNA oligomers were analyzed on a phosphorimaging scanner, STORM 820 [GE-Healthcare (Piscataway, NJ)].

DNA oligomers preparation. The phosphoramidite DNA monomer of Oxa and Oxa-containing DNA oligomers were prepared according to a chemical synthesis procedure previously reported [11]. Purification of synthetic DNA oligomers was performed with an RP-HPLC system using a gradient of CH_3CN using Eluent A [5% CH_3CN in 100 mM TEAA (pH 7.0)] and Eluent B [20% CH_3CN in 100 mM TEAA (pH 7.0)]; 15% (0 min)–80% (40 min) of Eluent B (flow rate: 1 ml/min). The DNA oligomers prepared in this study were listed in Table 1.

T4 polynucleotide kinase (T4 PNK) reaction. For kinetic analysis of PNK activity, DNA oligomers were prepared at different concentrations (1.5–15 nM), and incubated with T4 PNK (0.25 U) and [γ-^{32}P]ATP (66 μM) in 50 μl of reaction buffer [50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 5 mM DTT] at 37 °C. For each sample, two aliquots (10 μl) were taken from the reaction mixture at 1 min and 10 min and their reactions were terminated by heat deactivation (75 °C, 5 min). The reaction products were separated by 20% denaturing PAGE containing 6 M urea and the initial rate was estimated by comparing the product amounts of the samples. Phosphorylation efficiency was calculated by [(phosphorylated DNA substrates)/(Reference DNA)]. The sequence of the reference DNA was 5′-GAAACACTATTCCACGCGCCTTCTCTC-3′ (27mer). Relative phosphorylation efficiency of O-3′, A-3′, T-3′, and C-3′ was calculated by setting the phosphorylation efficiency of G-3′ to 100%.

T4 DNA ligase reaction. For preparation of the hot-labeled DNA oligomers, DNA samples (800 nM), especially down-stream ligation fragments (N-3′), were incubated with T4 PNK (40 U) and [γ-^{32}P]ATP (4.5 MBq) in 50 μl of reaction buffer at 37 °C for 30 min. The phosphorylation reaction was terminated by heat deactivation (75 °C, 10 min) and the hot-labeled DNA oligomer was separated using the purification column of CENTRI-SEP. Two fragments of 20mer DNA oligomers [one is up-stream DNA strand (5′-N; 600 nM) and the other down-stream DNA strand with 5′-end hot-label (N-3′; 600 nM)] and 40mer template DNA strand (3′-ZZ-5′; 600 nM) were incubated with T4 DNA ligase (5 U) in 60 μl of reaction buffer [50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA] at 37 °C for 15 min. The ligation reaction was terminated by heat deactivation (75 °C, 10 min) and the reaction products were separated by 20% denaturing PAGE containing 6 M urea. The ligation efficiencies were estimated by [(amount of product)/(amount of product + amount of free DNA)].

Restriction enzyme reaction. Hot-labeled DNA oligomers were prepared using the same method described in the *T4 DNA ligase reaction* section. 24mer DNA oligomers, which included the recognition sequence of restriction enzymes, with the 5′-end hot-labeled strand (600 nM) and 24mer template DNA strand (600 nM) were incubated with restriction enzymes (BamHI, BglII, EcoRI, EcoRV, and HindIII) (2 U) in 50 μl of reaction buffer [BamHI, EcoRV, HindIII: 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, BglII: 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, EcoRI: 5 mM Tris–HCl (pH 7.5), 10 mM NaCl, 1 mM $MgCl_2$] at 37 °C for 1 h. The reaction was terminated by heat deactivation (80 °C, 10 min) and the reaction products were separated by 20% denaturing PAGE containing 6 M urea. The cleavage efficiencies were estimated by [(amount of product)/(amount of product + amount of free DNA)].

Table 1
DNA oligomers used in this study.

Oligomer name	Sequence	cf
N-3′	5′-d(N CCAT TCCTG ATTCT AAGTG)-3′ N = G, A, C, T, O	T4 PNK or T4 DNA ligase
5′-N	5′-d(CTCAG GTCGA CAGTC TGCG N)-3′ N = G, A, C, T, O	T4 DNA ligase
3′-ZZ-5′	3′-d(GAGTC CAGCT GTCAG ACGC ZZ GGTA AGGAC TAAGA TTCAC)-5′ ZZ = CC, CT, CG, CA	T4 DNA ligase
RE-sense	5′-d(GAGTG CGGC NNNNNN GCGGC TCAG)-3′ NNNNNN=(G, O, A)(G,O,A)ATCC for BamHI A(G, O, A)ATCT for BglII (G, O, A)AATTC for EcoRI (G, O, A)ATATC for EcoRV AA(G, O, A)CTT for HindIII	Restriction endonuclease
RE-anti	3′-d(CTCAC GCCG ZZZZZZ CGCCG AGTC)-5′ ZZZZZZ = CCTAGG for BamHI TCTAGA for BglII CTTAAG for EcoRI CTATAG for EcoRV TTCGAA for HindIII	Restriction endonuclease

Note: O, oxanine.

Results and discussion

The effect of Oxa at the 5′-end of a single stranded DNA on the phosphorylation efficiency of the T4 polynucleotide kinase

The phosphorylation performance of the T4 polynucleotide kinase (T4 PNK), which catalyzes the transfer of γ-phosphate from ATP (co-factor) to the 5′-OH group of nucleoside or oligonucleotide, has been shown to be dependent on the 5′-end nucleobase (5′-end N; N = G, A, T and C) in single stranded DNA [12]. A previous report showed that the order of phosphorylation efficiency was 5′-end G > 5′-end T ∼ 5′-end A > 5′-end C [12]. In addition, X-ray crystallography data, which revealed the structure of T4 PNK complexed with several single stranded DNA, indicated that the mechanism of T4 PNK binding to a single stranded DNA varied depending on the 5′-end N of the DNA [13]. Based on these previous reports, we compared the phosphorylation performance of T4 PNK when an Oxa base was on the 5′-end of single stranded DNA to when other nucleobases were placed on the 5′-end of single stranded DNA.

As listed in Table 1, five kinds of synthetic oligodeoxynucleotides containing different 5′-end N (N-3′; N = O, G, A, T and C) were prepared and employed as substrates to assess their influence

on the 5′-OH kinase function of T4 PNK. As shown in Fig. 1, T4 PNK showed a slightly higher phosphorylation performance when O-3′ was used compared to when G-ODN was used. The order of the phosphorylation efficiencies regarding nucleobase types was as follows; 5′-end O > 5′-end G > 5′-end T ~ 5′-end A > 5′-end C. As listed in Table 2, the K_m and relative-V_{max} values were 1.55 ± 0.15 μM^{-1} and 0.88 ± 0.16, respectively, for O-3′, while these values were 2.43 ± 0.09 μM^{-1} and 1.00, respectively, for G-3′. These results indicated that, when Oxa was located at the 5′-end of single stranded DNA, T4 PNK could recognize and bind to the Oxa base as efficiently as to the Gua base.

Compared to natural bases (G, A, T and C), the Oxa base displayed no distinctive difference in terms of its molecular influence on the function of other DNA enzymes such as nuclease S1, exonuclease I, and several phosphatases [14]. These findings, in addition to the T4 PNK results described above, indicate that the molecular recognition of Oxa is very similar to those of the natural bases found in cellular system, in particular, to Gua; thus, it is highly likely that Oxa participates in the biological mechanisms involved in nucleoside or nucleotide metabolisms.

The effect of Oxa–Cyt base-pairing at the 3′-end of up-stream DNA strands on the ligation performance of the T4 DNA ligase

T4 DNA ligase catalyzes the formation of a phosphodiester linkage between juxtaposed 3′-OH end of an up-stream DNA fragment and 5′-phosphate end of a down-stream DNA fragment using ATP as a co-factor. In the T4 DNA ligase reaction, the B-to-A transition of the DNA conformation has been previously shown to occur locally at the linkage region encircled by the T4 DNA ligase and this B-to-A conformational change is believed to be essential for the ligation reaction process to occur [15,16]. Thus, the efficiency of the T4 DNA ligase reaction will depend on the type of 3′-end base-pairing between the up-stream DNA fragment and template [17]. For example, the ligation performance of the T4 DNA ligase decreases when the base at the 3′-end of the up-stream DNA fragment is incorrectly matched. Due to these properties of the T4 DNA ligase, we investigated the effect of Oxa:Cyt pairing (O:C) (in 3′-end of up-stream DNA strand) on the ligation performance of the T4 DNA ligase, and compared this response to that of G:C.

Various combinations of DNA ligation experiments were carried out to examine the effect of the type of 5′-end base-pairs of down-stream DNA strands such as a correct match and incorrect match. As shown in Table 3, the DNA ligation reactions in which the O:C pair between the 3′-end of the up-stream fragment and template was used were found to display slightly lower efficiencies compared to those of G:C. The relative values of the ligation efficiency of O:C to that of G:C were 70.7–85.9% according to the types of 5′-end base-pairs of down-stream DNA strands, as listed in Table 3. In addition, we compared the effect of an O:C pair to a G:C at the 3′-end of up-stream DNA strands on DNA ligation when the 5′-end base-pairs of down-stream DNA strands were incorrectly matched. Under these conditions, the relative values of the ligation efficiency of O:C to that of G:C were 70.7–73.1%, as shown in Table 3.

Since the T4 DNA ligase employed here has the ability to successfully carry out ligation even when the base-pairs are incorrectly matched, in particular the G:T mis-match, the high ligation performance observed when the O:C pair was employed may have resulted from the ability of the T4 DNA ligase to function even in the presence of mis-matches. To remove the possibilities of such artifacts, the *Taq* DNA ligase, whose ligation performance is highly-dependent on the accuracy of base-pairing at the 3′-end of the up-stream DNA strand and has been used to discriminate incorrect and correct base pair matches between DNA strands, was used in the ligation reactions. As shown in Fig. 2, when an O:C pair at the 3′-end of up-stream DNA strands was used, the DNA ligation efficiencies were 43% of those when the G:C pair

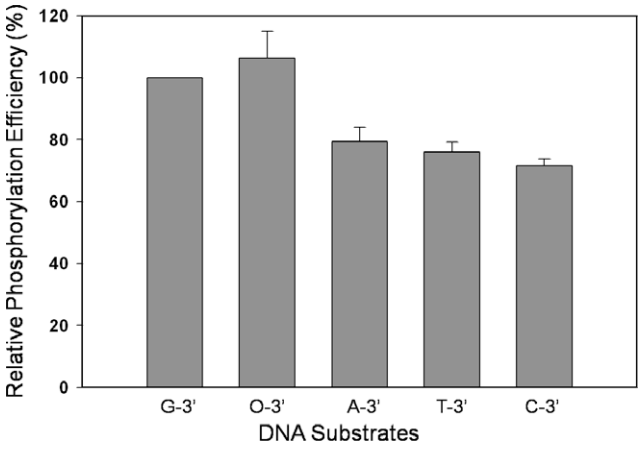

Fig. 1. Phosphorylation efficiencies of single stranded DNA by T4 polynucleotide kinase. Phosphorylation efficiency = [(phosphorylated DNA strands)/(Reference DNA)]. The sequence of reference DNA was 5′-GAAACACTATTCCACGCGCC TTCTCTC-3′ (27mer). Relative phosphorylation efficiencies of O-3′, A-3′, T-3′, and C-3′ were calculated by setting the phosphorylation efficiency of G-3′ to 100%. The product was separated by 20% denaturing PAGE containing 6 M urea. Values indicate mean ± SD for three independent experiments.

Table 2
Steady-state kinetic values for the phosphorylation reaction by T4 polynucleotide kinase.

DNA substrate	Relative-V_{max}[b]	K_m (mM^{-1})	Relative-V_{max}/K_m (mM^{-1})
G-3′[a]	1.00	2.43 ± 0.09[c]	0.41 ± 0.02
O-3′	0.88 ± 0.16	1.55 ± 0.15	0.58 ± 0.05

[a] DNA sequences for each substrates were listed in Table 1.
[b] Relative-V_{max} for O-3′ was estimated by dividing V_{max} of O-3′ by that of G-3′.
[c] Values indicate mean ± SD for three independent experiments.

Table 3
T_m values and relative ligation efficiencies for each substrate used in the enzyme reaction with T4 DNA ligase.

Template DNA[a]	Ligation fragments[a]	T_m[b] (°C)	Relative ligation efficiency[c] (%)
Matched case at 5′-end of down-stream			
3′-CC-5′	5′-G, G-3′	78.19, 69.40	100
	5′-O, G-3′	75.09, 69.40	85.9 ± 2.2
3′-CG-5′	5′-G, C-3′	77.84, 67.78	100
	5′-O, C-3′	75.53, 67.78	70.7 ± 9.3
3′-CA-5′	5′-G, T-3′	78.40, 65.30	100
	5′-O, T-3′	75.59, 65.30	74.1 ± 7.4
3′-CT-5′	5′-G, A-3′	78.14, 67.80	100
	5′-O, A-3′	76.62, 67.80	84.9 ± 3.1
Mis-matched case at 5′-end of down-stream			
3′-CG-5′	5′-G, T-3′	77.84, 65.70	100
	5′-O, T-3′	75.53, 65.70	70.7 ± 4.8
3′-CT-5′	5′-G, G-3′	78.14, 66.87	100
	5′-O, G-3′	76.62, 66.87	73.1 ± 8.5
3′-CG-5′	5′-G, A-3′	77.84, 64.75	No reaction
	5′-O, A-3′	75.53, 64.75	No reaction
3′-CA-5′	5′-G, G-3′	78.40, 64.75	No reaction
	5′-O, G-3′	75.59, 64.75	No reaction

[a] DNA sequences for each substrates were listed in Table 1.
[b] T_m was measured in case that the total concentrations of ODNs is 4 mM.
[c] Relative ligation efficiency was calculated by regarding the ligation efficiency for 5′-G as 100%.

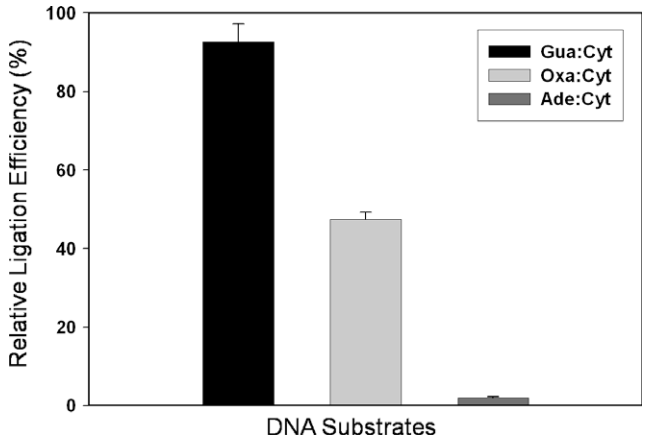

Fig. 2. Relative ligation efficiencies of DNA strands by *Taq* DNA ligase. Ligation efficiency = [(amount of the product)/(amount of the product + free DNA)]. DNA substrates used in this study were listed in Table 1. The product was separated by 20% denaturing PAGE containing 6 M urea. Values indicate mean ± SD for three independent experiments.

was used. In contrast, when the A:C pair was used, no ligation product was observed. These results indicated that O:C base-pairing in DNA stands was not as mis-matched as A:C pairing.

The effect of Oxa–Cyt base-pairing at the middle of DNA strands on the recognition of restriction endonuclease enzymes

We also analyzed the hydrolytic functions of restriction enzymes, which cleave specific DNA sequence, to assess the effect of Oxa:Cyt base-pairs in the middle of DNA strands on restriction enzyme recognition. In these experiments, BamHI, BglII, EcoRI, EcoRV, and HindIII were employed.

As shown in Fig. 3, DNA duplexes containing Oxa:Cyt were cleaved by restriction enzymes, while DNA duplexes containing Ade:Cyt mis-matched pair were not cleaved. It should be noted that Oxa has a similar structure to Gua, where N1 is substituted with oxygen. Thus, in regards to the major groove and minor groove of the DNA duplex, the environment encountered by the amino acid residues of DNA-binding proteins or DNA-recognizing

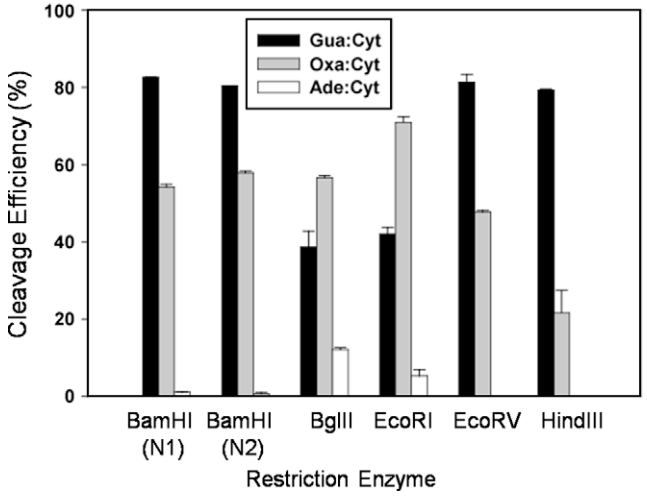

Fig. 3. Cleavage efficiencies of DNA duplexes by restriction endonuclease enzymes. Cleavage efficiency = [(amount of the product)/(amount of the product + free DNA)]. DNA substrates used in this study were listed in Table 1. The product was separated by 20% denaturing PAGE containing 6 M urea. Values indicate mean ± SD for three independent experiments.

enzymes may be similar between Oxa and Gua. Based on these considerations, Oxa could interact with DNA-relevant enzymes through a similar mechanism as the normal base, Gua.

One of the more interesting results of these experiments was that BglII and EcoRI showed much higher cleavage efficiencies for DNA duplexes containing Oxa:Cyt than normal DNA duplexes (154% and 164% higher efficiency, respectively). In a recent study, we found the flexibility and bending of the DNA substrate are important factors for DNA recognition by BglII and EcoRI [18]. Molecular modeling data also showed that O:C base pair is in equilibrium between the "Watson–crick" and "Wobble" conformations [6]. Therefore, Oxa base-pairing, itself, could contribute to a flexible conformational change in the DNA substrate, so that, the bending of the DNA substrate, which is a critical factor for efficient recognition by BglII or EcoRI, might be induced more effectively. In contrast, BamHI, EcoRV, and HindIII showed a slightly lower cleavage efficiency for DNA duplexes containing Oxa:Cyt than normal DNA duplexes, but the cleavage efficiency was still much higher than those of other mis-matches such as Ade:Cyt. It should be noted that to more precisely understand the effect of Oxa on the recognition mechanism of restriction enzymes, crystal complex structures between the restriction enzyme and Oxa-containing DNA substrate should be determined. However, the presence of Oxa may increase the DNA binding and recognition by some restriction enzymes, which required high flexibility and bending of the DNA substrate.

Conclusion

Oxa has received much attention in terms of its biological significance in cellular systems because Oxa can be generated from Gua by NO- or HNO_2-induced nitrosative oxidation. Since Oxa has been shown to mediate GC-to-AT transversion events during replication, Oxa is considered a mutagenic lesion. In addition, BER enzymes have been shown not to effectively recognize Oxa in DNA strands [7,9]; thus, there is a high probability that Oxa participates in biomolecular interactions related to DNA mechanisms. In particular, determining the biomolecular response patterns of Oxa to DNA-binding proteins or DNA-recognizing enzymes would provide important biophysical and biochemical clues for understanding the *in vivo* behavior of Oxa and its genotoxicity in cellular systems.

In this study, we examined the interaction of Oxa with different enzymatic proteins to understand how similarly it can participate in biomolecular interactions compared to the natural base, Gua. We designed several types of synthetic Oxa–ODNs, in which Oxa was located at the ends or middle of ODNs, and the prepared ODNs were used as substrates for several DNA-relevant enzymes. We employed three types of DNA-binding/recognizing enzymes as model systems. (1) T4 polynucleotide kinase (T4 PNK), (2) T4 DNA ligase, and (3) several restriction endonuclease enzymes (BamHI, BglII, EcoRI, EcoRV, and HindIII).

In the case of T4 PNK, a high phosphorylation efficiency was observed when Oxa was located on the 5′-end of single stranded DNA compared to when other nucleobases were located on the 5′-end of single stranded DNA. The order of phosphorylation efficiency was as follows: Oxa > Gua > Ade ~ Thy > Cyt. In the case of T4 DNA ligase, base-pairings of Oxa–Cyt between DNA fragments and template was found to show a lower ligation performance than natural base-pairing, Gua:Cyt, but this match was not considered as dramatic as a mis-match of Ade:Cyt with regards to ligation performance. In the case of restriction endonuclease enzymes, EcoRI and BglII showed higher cleavage activities on DNA substrates containing Oxa:Cyt pair than those containing Gua:Cyt, while BamHI, HindIII and EcoRV showed lower cleavage activity; however, this

decreased activity was relatively small. These results suggest that Oxa, once produced in a genome sequence, might participate in molecular and enzymatic reactions in a similar manner as the natural nucleobase, Gua.

This is the first study to examine the interactions between Oxa, located at the ends or in the middle of DNA strands, and DNA-relevant enzymes. These data could be useful for better understanding the biological significance of Oxa. As observed in this study, the activity of Oxa was similar to that of the natural base, Gua, in regards to its molecular interactions with DNA-binding/recognizing enzymes. However, Oxa does have different properties from Gua; thus, a subtle comparison between Oxa and Gua in DNA oligomers may provide useful clues for elucidating the molecular interaction mechanisms between DNA strands and DNA-binding/recognizing enzymes.

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