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Comparison of the molecular influences of NO-induced lesions in DNA strands on the reactivity of polynucleotide kinases, DNA ligases and DNA polymerases

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Nitric oxide (NO) causes DNA damage, generating xanthine (Xan, X) and oxanine (Oxa, O) from guanine (Gua, G) and hypoxanthine (Hyp, H) from adenine (Ade, A) by nitrosative oxidation. Although these NO-induced lesions have been thought to cause mutagenic problems in cellular systems, the influence of these lesions on enzymatic functions has not yet been compared systematically. In this study, we investigated the effect of NO-induced lesions on the activities of DNA-binding/recognizing enzymes such as T4 polynucleotide kinase (T4 PNK), DNA ligases (T4 DNA ligase, *Taq* DNA ligase) and DNA polymerases (*E. coli* DNA polymerase I, Klenow fragment, T4 DNA polymerase). The phosphorylation efficiencies of T4 PNK are dependent on the base type at the 5′-end of single-stranded DNA, where Oxa ≅ Hyp ≅ Gua > Xan ≅ Ade. The enzymatic reactions efficiencies of DNA ligases or DNA polymerases were observed to be dependent on the base-pairing type bound by the enzymes, where G:C > H:C > O:C > X:C and A:T ≅ H:T > O:T > X:T. These results suggested that NO-induced lesions and their base-pairs could participate in the interaction mechanisms of the DNA-binding/recognizing enzymes in a similar manner as natural nucleobases.

Keywords: DNA ligase/DNA polymerase/Nitric oxide (NO)-induced lesions/polynucleotide kinase.

Abbrreviations: Ade (A), adenine; Cyt (C), cytosine; DPC, DNA–protein cross-link; Gua (G), guanine; Hyp (H), hypoxanthine; ODN, oligodeoxynucleotide; Oxa (O), oxanine; PNK, polynucleotide kinase; Thy (T), thymine; T_m, melting temperature; Xan (X), xanthine.

Introduction

While xanthine (Xan, X) has been shown to be a major product of nitric oxide (NO)- or nitrous acid (HNO_2)-induced oxidative deamination of guanine (Gua, G), we previously showed that oxanine (Oxa, O) is also a product of these reactions (*1*). In this previous study, we demonstrated that Oxa could form together with Xan at a molar ratio of 1:3 when 2′-deoxyguanosine or DNA was treated with NO or weakly acidic HNO_2. In addition, nitrosative oxidation (deamination) of adenine (Ade, A) also leads to the formation of hypoxanthine (Hyp, H) (*2*). These NO-induced lesions (Xan, Oxa and Hyp) are expected to make base-pairs both with cytosine (Cyt, C) and thymine (Thy, T) through two hydrogen bonds (Fig. 1); thereby causing severe genotoxic problems such as G:C→A:T (for Xan or Oxa instead of Gua) or A:T→G:C (for Hyp instead of Ade) transversion (*3–11*). Moreover, Oxa can induce DNA–protein cross-link (DPC) formation with some DNA-binding enzymes or proteins, leading to other genotoxic or cytotoxic events in cellular systems (*12*). The biophysical and biochemical properties of NO-induced lesions have been extensively investigated (*6*, *11*, *13–18*). However, there have been no systematic studies on the molecular effects of NO-induced lesions on DNA-relevant enzymes.

Hyp-containing oligodeoxynucleotide (Hyp-ODN) is commercially provided and Xan-ODN can be prepared using previously established chemical synthesis methods (*11*). Recently, we have developed a chemical synthesis procedure for obtaining Oxa-ODN (*13*). Therefore, due to the availability of Hyp-, Xan- and Oxa-ODN, we can systematically investigate their influence on enzymatic functions. In this study, we prepared several types of synthetic Xan-, Oxa- and Hyp-ODNs where the lesions were located at the end of synthetic ODNs. These lesion-containing ODNs were then used as substrates for several DNA-binding/recognizing enzymes to systematically compare the effects of Xan, Oxa and Hyp in DNA strands on enzymatic functions. The DNA-binding/recognizing enzymes, T4 polynucleotide kinase (T4 PNK), DNA ligase (T4 DNA ligase, *Taq* DNA ligase) and DNA polymerases (*Escherichia coli* DNA polymerase I, Klenow fragment, T4 DNA polymerase) were used in these studies. These findings will be useful for understanding the biomolecular interaction behavior of damaged or modified DNA bases, which may lead to genetic problems in cellular systems.

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 PNK, T4 DNA ligase, *Taq* DNA ligase, T4 DNA polymerase, *E. coli* DNA polymerase I and Klenow fragment were acquired from Takara (Shiga, Japan). Other chemical reagents were purchased from Wako (Osaka, Japan) and solvents from Nacalai Tesques (Osaka, Japan).

DNA oligomer preparation

Normal DNA oligomers and Hyp-ODNs were synthesized through Sigma-Aldrich Japan (Tokyo, Japan). Xan- and Oxa-ODNs were prepared according to previously published chemical synthesis procedures (*11*, *13*). Purification of synthetic DNA oligomers was performed with an RP-HPLC system using a gradient of CH_3CN with Eluent A [5% CH_3CN in 100 mM triethylammonium acetate (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 presence of Oxa and Xan in DNA oligomers were confirmed by enzymatic digestion (*13*). The DNA oligomers prepared in this study were listed in Table I.

Preparation of hot-labelled DNA oligomer

For preparation of the hot-labeled DNA oligomers, single-stranded DNA samples (800 nM) were incubated with T4 PNK (40 unit) and [γ-^{32}P]ATP (4.5 MBq) in 50 µl of reaction buffer [50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 5 mM dithiothreitol (DTT)] at 37°C for 30 min. The reaction was terminated by heat deactivation (75°C, 10 min) and the hot-labeled DNA oligomers were separated using the CENTRI-SEP purification column.

Polynucleotide kinase assay

For enzymatic analysis of PNK activity, single-stranded DNA oligomers with different 5′-end nucleobases (N-3′, 5 nM) were prepared and incubated with T4 PNK (0.25 unit) and [γ-^{32}P]ATP (66 µM) in 50 µl of reaction buffer at 37°C. For each sample, aliquots (10 µl)

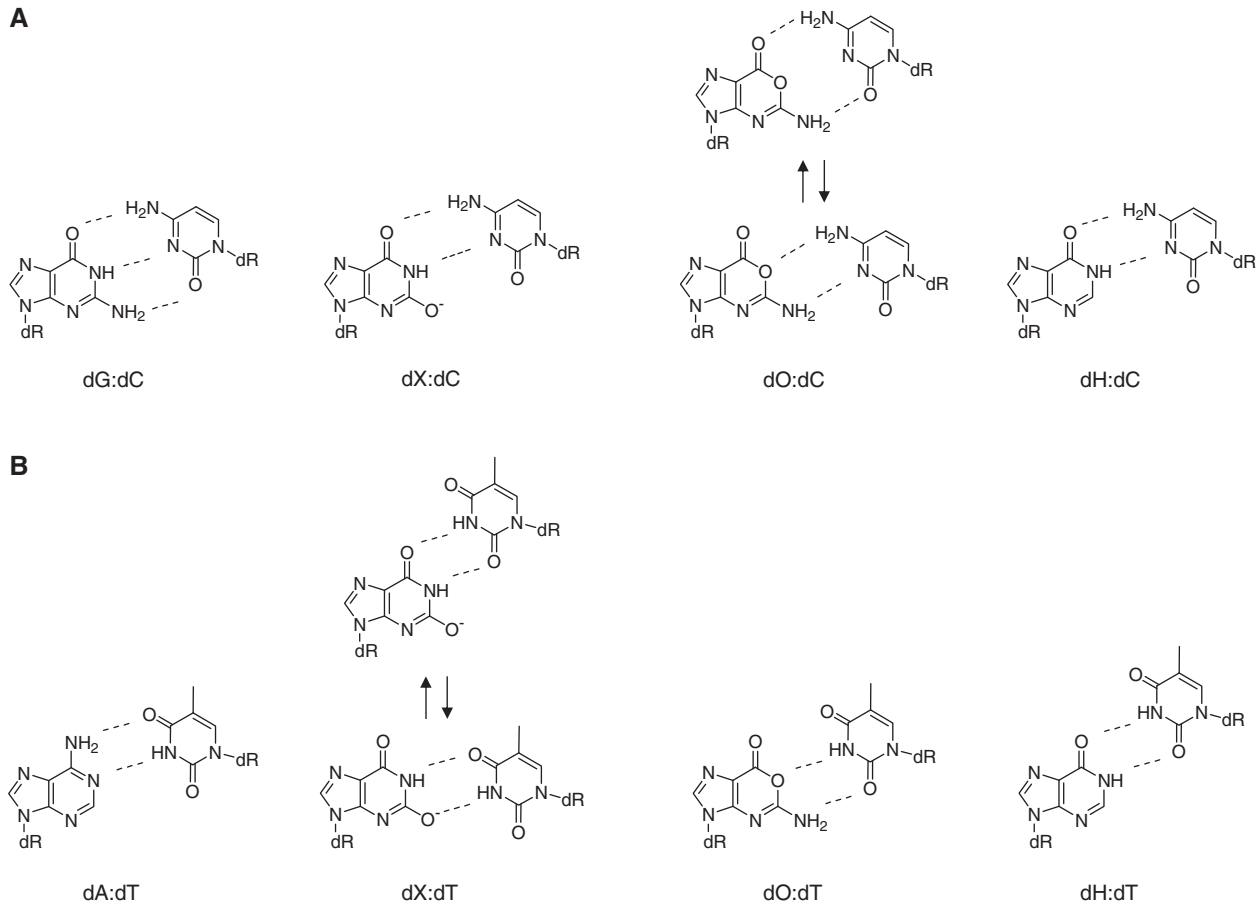

Fig. 1 Proposed base-pairing structures of Gua (A), Ade (B) and NO-induced lesions paired with Cyt (A) or Thy (B). Dashed line indicates the hydrogen bonds.

Table I. DNA oligomers used in this study.

Name	DNA oligomer	Cf
N-3′	5′-d(N CCAT TCCTG ATTCT AAGTG)-3′ N = G, A, X, O, H	Substrate for polynucletide kinase Fragment for DNA ligases
5′-N	5′-d(CTCAG GTCGA CAGTC TGCG N)-3′ N = G, A, X, O, H	Fragment for DNA ligases Primer for DNA polymerases
3′-Z_1Z_2-5′:	3′-d(GAGTC CAGCT GTCAG ACGC Z_1Z_2GGTAAGGAC TAAGA TTCAC)-5′ Z_1Z_2 = CC, CT, TC, TT	Template for DNA ligases and DNA polymerases

X: Xanthine, O: Oxanine, H: Hypoxanthine.

were taken from the reaction mixture at 5 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 amount of product was quantified using a phosphor-imaging scanner, STORM 820 [GE-Healthcare (Piscataway, NJ)]. The relative phosphorylation efficiencies were estimated by the ratio of (amount of the product)/(amount of the reference DNA). The sequence of the reference DNA was 5′-GAAA CACTATTCCACGCGCCTTCTCTC-3′ (27mer). For kinetic analysis of PNK activity, DNA oligomers were prepared at different concentrations (1.5, 3, 5, 7.5, 10, 15 nM). Michaelis constants (K_m) and maximum rates of reaction (V_{max}) were obtained from Lineweaver–Burk plots.

DNA ligase assays

Two fragments of 20mer DNA oligomers [up-stream DNA strand (5′-N, 600 nM) and down-stream DNA strand hot-labeled at the 5′-end (N-3′, 600 nM)] and 40 mer template DNA strand (3′-Z_1Z_2-5′, 600 nM) were incubated with T4 DNA ligase (5 unit) or *Taq* DNA ligase (5 unit) in 60 µl of reaction buffer [for T4 DNA ligase, 66 mM Tris–HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT; for *Taq* DNA ligase, 20 mM Tris–HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD, 0.1% Triton X-100] at 16°C (for T4 DNA ligase) or 45°C (for *Taq* DNA ligase) for 15 min, respectively. The reactions were terminated and the amount of product was quantified using a phosphor-imaging scanner, STORM 820. The ligation efficiencies were estimated by: (amount of product)/(amount of product + amount of free DNA).

DNA polymerase assays

Up-stream DNA strands hot-labeled at the 5′-end (5′-N, 600 nM) and 40 mer template DNA strands (3′-Z_1Z_2-5′, 600 nM) were incubated with dNTP mixture (200 µM) and *E. coli* DNA polymerase I (1 unit), Klenow fragment (1 unit), or T4 DNA polymerase (1 unit), in 60 µl of reaction buffer [for *E. coli* DNA polymerase I, 50 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 100 µl DTT, 0.025% bovine serum albumin; for Klenow fragment, 10 mM Tris–HCl (pH 7.5), 7 mM $MgCl_2$, 100 µM DTT; for T4 DNA polymerase, 33 mM Tris–acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 500 µM DTT, 0.1% BSA.] at 37°C for 5 min. The reactions were terminated and the amount of product was quantified using a phosphor-imaging scanner, STORM 820. The polymerization efficiencies were estimated by: (amount of product)/(amount of product+ amount of free DNA).

Melting temperature analysis

For melting temperature analysis, DNA solutions (2 µM) were prepared in a phosphate buffer (1 M NaCl, 10 mM Na_2HPO_4 and 1 mM Na_2EDTA adjusted to pH 7.0 with HCl). Absorbance of solutions containing a 1:1 strand ratio of oligodeoxynucleotides at 260 nm was obtained using a Shimadzu TMSPC-8 T_m analysis system where the temperature was increased from 20 to 90°C at a rate of 0.2°C min^{-1}.

Results

The reaction efficiencies of T4 polynucleotide kinase on the NO-induced lesions

As listed in Table I, five kinds of synthetic oligodeoxynucleotides containing different 5′-end nucleobases (N-3′; N = G, A, X, O, H) were prepared and employed as substrates to assess their influences on the 5′-OH kinase functions of T4 PNK. The phosphorylation performance of T4 PNK has been previously shown to be dependent on the 5′-end nucleobase in single-stranded DNA (*19*). It has been shown that the amino acids of T4 PNK involved in the recognition of single-stranded DNA is dependent on the type of 5′-end nucleobase (*20*). As shown in Fig. 2, the order of

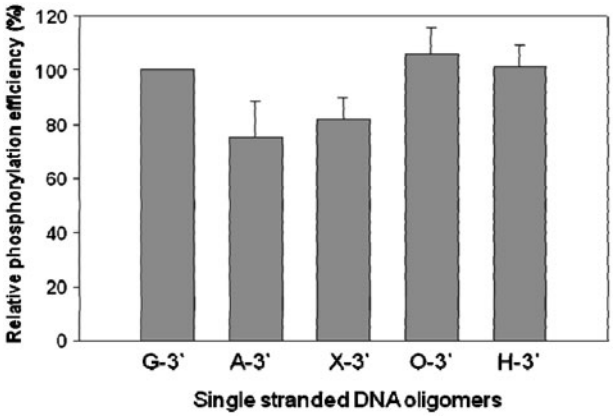

Fig. 2 Phosphorylation efficiencies of T4 polynucleotide kinase for single-stranded DNA. Phosphorylation efficiency is [phosphorylated DNA substrates]/[Reference DNA (27 mer: 5′-GAAACACTATTC CACGCGCCTTCTCTC-3′)]. Relative phosphorylation efficiency of A-3′, X-3′, O-3′ and H-3′ were calculated as the relative phosphorylation efficiency 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 II. Steady-state kinetic values for T4 polynucleotide kinase reactions.

DNA substrate[a]	Relative−V_{max}[b]	K_m (μM^{-1})[b]	Relative−V_{max}/K_m (μM^{-1})[b]
G-3′	1.001	2.43 ± 0.09	0.41 ± 0.02
A-3′	0.84 ± 0.13	2.33 ± 0.34	0.37 ± 0.08
X-3′	0.70 ± 0.08	1.83 ± 0.14	0.38 ± 0.03
O-3′	0.88 ± 0.16	1.55 ± 0.15	0.58 ± 0.05
H-3′	0.80 ± 0.14	1.55 ± 0.16	0.52 ± 0.06

[a]DNA sequences for each substrate were listed in Table I.
[b]Values indicate mean ± SD for three independent experiments.

the phosphorylation efficiencies observed in this study was O-3′ ≅ H-3′ ≅ G-3′ > X-3′ ≅ A-3′. The K_m and V_{max} values, which are basic parameters used to understand the properties of enzyme reactions, were also investigated (Table II). The estimated K_m and relative-V_{max} values (setting the value of G-3′ to 1.00) were as follows: 2.43 ± 0.09 μM^{-1} and 1.00 (for G-3′), 2.33 ± 0.34 μM^{-1} and 0.84 ± 0.13 (for A-3′), 1.83 ± 0.14 μM^{-1} and 0.70 ± 0.08 (for X-3′), 1.55 ± 0.15 μM^{-1} and 0.88 ± 0.16 (for O-3′), and 1.55 ± 0.16 μM^{-1} 0.80 ± 0.14 (for H-3′) (Table II).

The reaction efficiencies of DNA ligases and DNA polymerases on the NO-induced lesions

The reaction efficiencies of several DNA ligases and DNA polymerases were investigated for DNA substrates with different base-pairs (Tables III and IV). Both the relative ligation efficiencies and polymerization efficiencies on DNA substrates containing Hyp:Cyt pair (dsH:C) were less than normal DNA duplexes (dsG:C) with some exceptions (such as dsH:C > dsG:C in *Taq* DNA ligase and Klenosw fragment). In addition, both the ligation efficiencies and polymerization efficiencies on dsO:C were less than those of dsH:C, and the efficiencies for dsX:C were

Table III. Relative ligation efficiency for the enzymatic reactions of DNA ligases

Type	Substrates[a]	N	Relative ligation efficiency (%)[b,c] T4 DNA ligase	Relative ligation efficiency (%)[b,c] *Taq* DNA ligase	Substrates[a]	N	Relative ligation efficiency (%)[b,c] T4 DNA ligase	Relative ligation efficiency (%)[b,c] *Taq* DNA ligase
dsN:C	5′-N, G-3′	G	100	100	5′-G, N-3′	G	100	100
	3′-CC-5′	X	87.2 ± 0.5	78.2 ± 4.3	3′-CC-5′	X	99.7 ± 0.1	107.0 ± 4.6
		O	85.9 ± 2.2	82.8 ± 3.3		O	101.5 ± 0.5	103.4 ± 1.9
		H	93.8 ± 1.5	92.2 ± 2.2		H	98.6 ± 0.2	107.0 ± 4.4
	5′-N, A-3′	G	100	100	5′-A, N-3′	G	100	100
	3′-CT-5′	X	90.6 ± 0.6	78.7 ± 4.2	3′-TC-5′	X	96.3 ± 0.9	107.0 ± 1.3
		O	84.9 ± 3.1	87.2 ± 4.5		O	98.4 ± 0.6	102.7 ± 0.8
		H	94.1 ± 1.1	95.3 ± 4.5		H	97.8 ± 1.9	105.3 ± 1.3
dsN:T	5′-N, G-3′	A	100	100	5′-G, N-3′	A	100	100
	3′-TC-5′	X	97.3 ± 1.8	89.6 ± 1.6	3′-CT-5′	X	99.7 ± 0.5	98.3 ± 0.4
		O	89.4 ± 0.9	94.9 ± 4.8		O	103.1 ± 0.3	97.4 ± 0.9
		H	100.5 ± 1.1	105.8 ± 3.7		H	99.5 ± 0.2	103.3 ± 0.6
	5′-N, A-3′	A	100	100	5′-A, N-3′	A	100	100
	3′-TT-5′	X	96.8 ± 1.5	82.1 ± 4.2	3′-TT-5′	X	96.5 ± 1.2	105.7 ± 2.1
		O	90.6 ± 0.6	91.3 ± 2.7		O	100.7 ± 0.2	101.0 ± 1.6
		H	101.9 ± 2.0	92.3 ± 3.1		H	100.1 ± 0.8	100.8 ± 1.1

[a]DNA oligomer sequences for each substrate were listed in Table I.
[b]Values indicate mean ± SD for three independent experiments.
[c]Relative ligation efficiency was estimated by dividing each ligation efficiency by that of dsG:C or dsA:T.

Table IV. Relative polymerization efficiency for the enzymatic reactions of DNA polymerases

Type	Substrates[a]	*N*	Relative polymerization efficiency (%)[b,c] *E. coli* DNA polymerase I	Klenow fragment	T4 DNA polymerase
dsN:C	5′-N	G	100	100	100
	3′-CC-5′	X	65.0 ± 4.3	70.4 ± 3.0	79.7 ± 3.6
		O	89.6 ± 0.9	86.6 ± 4.4	88.6 ± 3.9
		H	95.9 ± 5.3	94.7 ± 4.8	90.3 ± 4.3
	5′-N	G	100	100	100
	3′-CT-5′	X	65.4 ± 5.2	63.1 ± 3.8	80.7 ± 4.3
		O	81.7 ± 3.5	84.2 ± 0.6	91.4 ± 5.0
		H	98.3 ± 5.2	106.4 ± 5.3	93.9 ± 3.8
dsN:T	5′-N	A	100	100	100
	3′-TC-5′	X	74.0 ± 5.6	94.1 ± 3.6	90.4 ± 3.4
		O	88.1 ± 6.1	97.9 ± 4.2	94.8 ± 3.3
		H	93.0 ± 5.7	116.6 ± 5.4	99.1 ± 6.1
	5′-N	A	100	100	100
	3′-TT-5′	X	62.1 ± 3.2	72.2 ± 5.7	75.9 ± 5.0
		O	82.9 ± 5.8	92.4 ± 5.8	79.4 ± 4.3
		H	93.1 ± 6.5	96.1 ± 4.0	93.5 ± 3.0

[a]DNA oligomer sequences for each substrate were listed in Table I.
[b]Values indicate mean ± SD for three independent experiments.
[c]Relative polymerization efficiency was estimated by dividing each polymerization efficiency by that of dsG:C or dsA:T.

Table V. Melting temperatures of DNA duplexes containing N:C or N:T base-pairing

Type	DNA strands	N	T_m (°C)[a]	ΔT_m (°C)[b]
dsN:C	5′-N, 3′-CC-5′	G	77.4	0
		X	74.8	−2.6
		O	74.8	−2.6
		H	76.0	−1.4
	5′-N, 3′-CT-5′	G	77.3	0
		X	74.8	−2.5
		O	75.4	−1.9
		H	76.2	−1.1
dsN:T	5′-N, 3′-TC-5′	A	75.9	0
		X	75.0	−0.9
		O	75.6	−0.3
		H	76.0	0.1
	5′-N, 3′-TT-5′	A	77.6	0
		X	76.6	−1.0
		O	76.9	−0.7
		H	77.9	0.3

[a]T_m was measured in case that total concentration of ODNs is 4 mM.
[b]ΔT_m was calculated by T_m(dsN:C)−T_m(dsG:C) or T_m(dsN:T)−T_m(dsA:T).

the lowest. It is worth noting that both the relative ligation efficiencies and polymerization efficiencies of dsH:T were almost the same to those of normal dsA:T, while those of dsX:T and dsO:T were less than those of dsG:T (even less than dsA:T or dsH:T).

It has been known that the stability of DNA base-pairing in the catalytic site is a critical factor in the reaction efficiencies of DNA ligases or DNA polymerases (*21–30*). Thus, the melting temperatures (T_m) of DNA substrates containing NO-induced lesions were compared to that of normal DNA substrate. As shown in Table V, both dsX:C and dsO:C showed low T_m values compared to dsG:C (even less than dsH:C). The stability of dsH:C was between that of normal dsG:C and dsX:C (or dsO:C). Meanwhile, dsX:T and dsO:T were less stable compared to normal dsA:T, while the stability of dsH:T was almost the same as normal dsA:T. The order of stability of DNA substrates considering T_m was as follow: dsG:C > dsH:C > dsO:C > dsX:C and dsA:T ≅ dsH:T > dsO:T >dsX:T. A similar trend was also observed for the relative ligation efficiencies of DNA ligases and polymerization efficiencies of DNA polymerases; dsG:C > dsH:C > dsO:C > dsX:C and dsA:T ≅ dsH:T > dsO:T > dsX:T with some exceptions (such as dsH:C > dsG:C in *Taq* DNA ligase and Klenow fragment).

Discussion

The effects of NO-induced lesions at the 5′-end of single-stranded DNA on the reaction of T4 polynucleotide kinase

T4 PNK catalyzes the transfer of γ-phosphate from ATP (co-factor) to the 5′-OH group of a nucleoside or oligonucleotide and its phosphorylation performance was known to be dependent on the 5′-end nucleobase in a single-stranded DNA. The degree to which the 5′-end nucleobase affects the catalytic activity of the T4 PNK enzyme was previously reported to be Gua > Thy ≅ Ade > Cyt (*19*). Analysis of several complex structures composed of T4 PNK and single-stranded DNA revealed by X-ray crystallography suggested that the residues at the binding pocket of T4 PNK, which interact with the 5′-end of single-stranded DNA, are different according to the 5′-end nucleobase (*20*).

In this study, DNA oligomers with NO-induced lesions or normal bases at the 5′-end were employed as substrates for T4 PNK. The order of phosphorylation efficiencies was as follows: Oxa ≅ Hyp ≅ Gua > Xan ≅ Ade. It is remarkable that T4 PNK showed the highest phosphorylation efficiency when Oxa was located at the 5′-end of single-stranded DNA (O-3′). All the tested nucleobases were purine bases. By comparing their structural conformations, it was inferred that the 6-carbonyl group in the purine-base ring might play a critical role in efficient recognition of T4 PNK, where Ade possessing an amino group (not a carbonyl group) showed the lowest phosphoryaltion efficiency among the tested purines. Interestingly, the interaction between single-stranded DNA and T4 PNK was not severely influenced by any of the NO-damaged lesions (Oxa, Hyp and Xan) at the 5′-end of DNA. Moreover, Oxa and Hyp showed a more preferable response. These results suggest that NO-induced lesions could participate in several nucleoside or nucleotide metabolism processes in the same way as normal bases when it is present in the genome.

The effect of DNA substrates containing NO-induced lesions at the 3′-end of up-stream DNA strands on the reactions of DNA ligases and DNA polymerases

It is known that the stability of the DNA base-pairs at the catalytic site of DNA ligases or DNA polymerases is strongly involved in the efficiencies of the enzymatic reactions (*21–30*). DNA ligases more interactively encircled the 5′-phosphorylated end of down-stream ligation fragments than the 3′-OH end of up-stream ligation fragments. Thus, the mismatched pairs at the 3′-end of up-stream ligation fragment and the template are more detrimental to ligation activity (*23*). As presented in Table III, all of the base-pairs at the 5′-end of the down-stream fragment, even when the NO-lesions were paired with Cyt or Thy, were successfully ligated for both the T4 DNA ligase and *Taq* DNA ligase. However, in cases for the base-pairs at the 3′-end of the up-stream ligation fragment (5′-N; N = G, A, X, O, H) and the template (3′-Z_1Z_2-5′: Z_1 = C or T), the efficiency of ligation was dependent on the stability between the up-stream fragment and template. The order of the ligation efficiencies on DNA substrate containing N:C or N:T (N = G, A, X, O, H) was G:C > H:C > O:C > X:C or A:T ≅ H:T > O:T >X:T. This order follows the same trend as the T_m order of the DNA duplexes. It is known that *Taq* DNA ligase is more sensitive to the mismatched pair at the ligation junction than T4 DNA ligase (*22*).

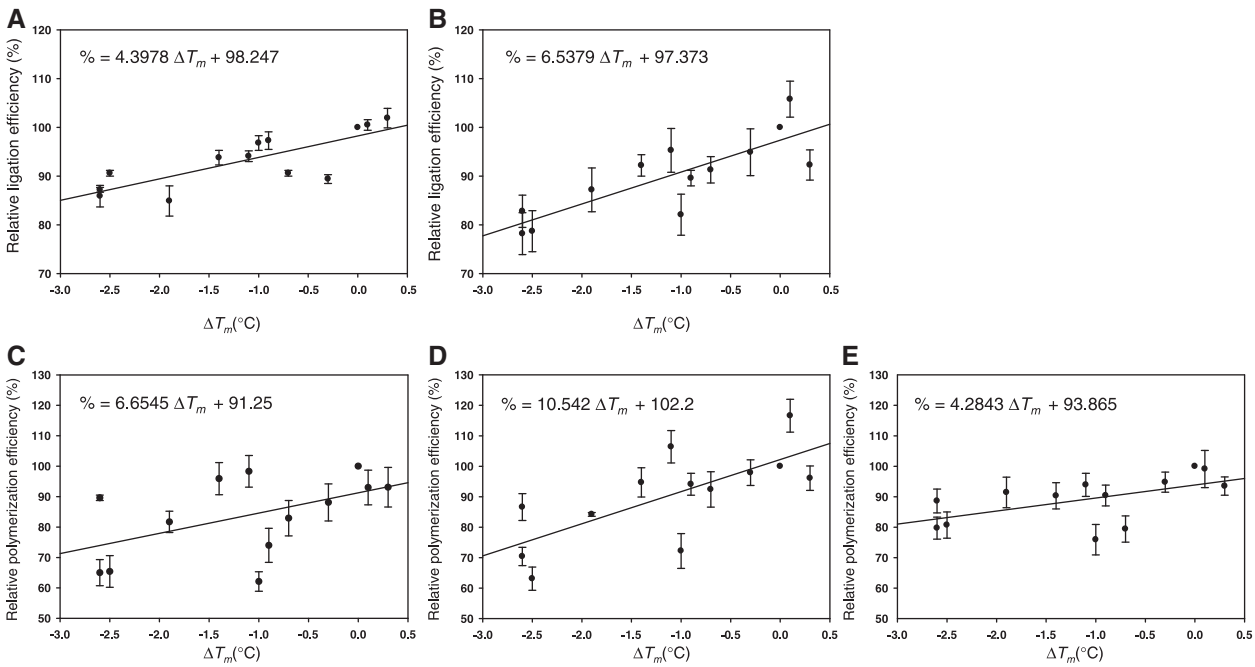

Fig. 3 Reaction efficiencies of DNA ligases [T4 DNA ligase (A), *Taq* DNA ligase (B)] or DNA polymerases [*E. coli* DNA polymerase I (C), Klenow fragment (D), T4 DNA polymerase (E)] according to ΔT_m values ($\Delta T_m = T_m$ of the normal DNA substrate $- T_m$ of the tested DNA substrate). The data for T_m and the relative ligation or polymerization efficiencies were listed in Tables 3–5.

As shown in Fig. 3A and B, *Taq* DNA ligase showed more decreased efficiency for less stable DNA substrates (with low T_m).

In the case of the polymerase chain elongation reaction, a similar tendency was observed for the dependency of the enzymatic efficiency on the stability of the DNA substrates. It has been known that the nucleotidyl transfer reactions catalyzed by DNA polymerases are based on a two-metal-ion mechanism (*25*) and the fidelity of the nucleotidyl transfer reaction is involved with the energetic and structural stabilities of the base pairs of DNA duplexes (*27*, *29*, *30*). As shown in Table IV, the order of the polymerization efficiencies on DNA substrates containing N:C or N:T (N = G, A, X, O, H) were as follows: G:C > H:C > O:C > X:C or A:T ≅ H:T > O:T >X:T, respectively. This follows the same trend observed for the T_m order of DNA duplexes. It is known that T4 DNA polymerases have higher 3′→5′ exonuclease activity (higher proofreading ability) than *E. coli* DNA polymerase I and Klenow fragment (*31*). As shown in Fig. 3C–E, T4 DNA polymerases showed better chain elongation performances for less stable DNA substrates (with low T_m) than the other DNA polymerases.

DNA duplexes containing NO-lesions are energetically and structurally less stable than normal DNA duplexes. Thus, the NO-lesions in DNA substrates could make an unfavorable effect on the catalytic mechanisms of DNA ligases or DNA polymerases, leading to low performances in ligation and polymerization reactions (Tables III–V and Fig. 3). However, the reaction efficiencies of DNA ligases and DNA polymerases for NO-induced lesions in DNA duplexes were not so severely decreased. These results indicate that there is a high possibility that NO-induced lesions could be processed together with normal bases by DNA-relevant enzymes.

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Conflict of interest
None declared.

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