

Analysis of DNA-Recognizing Enzyme Interaction using Deaminated Lesions

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Abstract—Deaminated lesions were produced via nitrosative oxidation of natural nucleobases; uracil (Ura, U) from cytosine (Cyt, C), hypoxanthine (Hyp, H) from adenine (Ade, A), and xanthine (Xan, X) and oxanine (Oxa, O) from guanine (Gua, G). Such damaged nucleobases may induce mutagenic problems, so that much attentions and efforts have been poured on the revealing of their mechanisms *in vivo* or *in vitro*. In this study, we employed these deaminated lesions as useful probes for analysis of DNA-binding/recognizing proteins or enzymes. Since the pyrimidine lesions such as Hyp, Oxa and Xan are employed as analogues of guanine, their comparative uses are informative for analyzing the role of Gua in DNA sequence in DNA-protein interaction. Several DNA oligomers containing such Hyp, Oxa or Xan substituted for Gua were designed to reveal the molecular interaction between DNA and protein. From this approach, we have got useful information to understand the molecular mechanisms of the DNA-recognizing enzymes, which have not ever been observed using conventional DNA oligomer composed of just natural nucleobases.

Keywords—Deaminated lesion, DNA-protein interaction, DNA-recognizing enzymes

I. INTRODUCTION

WHEN DNA was treated with NO or weakly acidic HNO₂, deaminated lesions are produced. Xanthine (Xan, X) is known to be the major domination product of guanine (Gua, G). Recently, we showed that oxanine (Oxa, O) is another product of such oxidation process of Gua [1]. It was also found that hypoxanthine (Hyp, H) is formed from oxidation of adenine (Ade, A) [2]. In similar mechanism, uracil (Ura, U) can be produced from cytosine (Cyt, C), thymine (Thy, T) from 5-methyl cytosine and so on. Among such NO-induced lesions, Xan, Oxa, and Hyp which are originated from natural pyrimidine bases are expected to make base-pairs both with natural Cyt and Thy through hydrogen bonding; thereby revoking severe genotoxic problems such as G:C→A:T (presence of Xan, Oxa instead of Gua) or A:T→G:C (presence of Hyp instead of Ade) transversions [3-8]. Interestingly, Oxa possesses *O*-acylisourea structure in the aromatic ring to mediate a cross-link formation with some DNA-binding enzymes or proteins, which is another genotoxic event in cellular systems [9-11]. Generally, the biophysical and biochemical properties of such pyrimidine lesions have been focused or investigated *in vivo* and *in vitro* in terms of their genotoxic/cytotoxic influences [6, 12-18].

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In other view-point, these lesions can be used for molecule-based analysis of DNA-binding/recognizing proteins or enzymes. Since the pyrimidine lesions such as Xan, Oxa and Hyp are employed as analogues of guanine, the comparative results from their usage can provide important or new information on the role of Gua in DNA sequence in DNA-protein interaction. Xan-ODN can be prepared using chemical synthesis method established previously [8] and Hyp-containing oligodeoxynucleotide (Hyp-ODN) is already commercially available and. In addition, we set up a solid-phase chemical synthesis procedure for preparing Oxa-ODN [12]. That is, several DNA oligomers containing such Xan, Oxa or Hyp, which are substituted for Gua, can be used for revealing the molecular interaction between DNA and protein. In this report, we employed these deaminated lesions as novel molecular bioprobes for analysis of DNA-recognizing enzymes. In particular, we show new research approach to investigate molecular mechanisms of DNA ligases and DNA polymerases using Xan-ODNs, Oxa-ODNs or Hyp-ODNs as probe molecules.

II. MATERIAL METHODS

A. Enzymes and Reagents

DNA ligases, DNA polymerases and T4 DNA polynucleotide kinase (T4 PNK) were acquired from NEB (Ipswich, MA). [γ -³²P]ATP was purchased from GE Healthcare (Piscataway, NJ). The reagents for oligodeoxynucleotide synthesis (including CPG column and appropriately protected normal nucleosides) were acquired from Glen Researches Co. (Sterling, VA). Other chemical reagents were purchased from Wako (Osaka, Japan) and solvents from Nacalai Tesques (Osaka, Japan).

B. Preparation of single DNA strands

Xan-ODNs and Oxa-ODNs were prepared according to previously published chemical synthesis procedures [8, 12]. Hyp-ODNs and normal DNA oligomers were synthesized and acquired commercially. The synthesized DNA oligomers were purified with an RP-HPLC system using a gradient of CH₃CN [Eluent A [5% CH₃CN in 100 mM triethylammonium acetate (TEAA) (pH 7.0)] and Eluent B [20% CH₃CN in 100 mM TEAA (pH 7.0)]; 15% (0 min)- 80% (40 min) of Eluent B (flow rate : 1 ml/min)]. The presence of Xan, Oxa and Hyp in the synthesized DNA oligomers was confirmed through enzymatic digestion [12]. The oligodeoxynucleotides prepared in this study were arranged in Table I.

C. Hot-labeled DNA oligomer preparation

The preparation procedure of the hot-labeled DNA oligomers is as follow; DNA oligomer samples (800 nM) were incubated with T4 PNK (40 unit) and [γ -³²P]ATP (4.5 MBq) in 50 μ l of reaction buffer [50 mM Tris-HCl (pH 7.5), 10 mM

MgCl₂ and 5 mM dithiothreitol (DTT)] at 37°C for 30 min. The enzyme reaction was terminated by heat deactivation (75°C, 10 min) and the hot-labeled DNA oligomers were separated using the CENTRI-SEP purification column.

TABLE I
OLIGODEOXYNUCLEOTIDES USED IN THIS STUDY

Name	DNA Sequence	cf
N-3'	5'-d(N CCAT TCCTG ATTCT AAGTG)-3'	N = G, A, X, O, H Primer for DNA polymerization Down-stream fragment for DNA ligation reaction
5'-N	5'-d(CTCAG GTCGA CAGTC TGCG N)-3'	N = G, A, X, O, H Up-stream fragment for DNA ligation reaction
3'-M ₁ M ₂ -5'	3'-d(GAGTC CAGCT GTCAG ACGC M ₁ M ₂ GGTA AGGAC TAAGA TTCAC)-5'	M ₁ M ₂ = CC, CT, TC, TT Template for DNA polymerization and DNA ligation

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

D. DNA polymerase assays

DNA oligomers hot-labeled at the 5'-end (5'-N, 600 nM) and template DNA oligomers (3'- M₁M₂-5', 600 nM) were incubated with dNTP mixture (200 μM) and DNA polymerase (1 unit) in 60 μl of reaction buffer [10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT] at 25°C for 5 min. The reactions were terminated by heat deactivation (75°C for 20 min) and the amount of product was investigated using a phosphor-imaging scanner, STORM 820. The polymerization efficiencies were estimated by [(amount of product)/(amount of product) + (amount of free DNA)].

E. DNA ligase assays

Up-stream DNA oligomer (5'-N, 600 nM), down-stream DNA oligomer hot-labeled at the 5'-end (N-3', 600 nM) and template DNA oligomer (3'- M₁M₂-5', 600 nM) were incubated with DNA ligase (5 unit) in 60 μl of reaction buffer [30 mM Tris-HCl (pH 8.0), 4 mM MgCl₂, 26 μM NAD, 1 mM DTT, 50 μg.ml BSA] at 16°C for 15 min. The reactions were terminated by heat deactivation (65°C for 20 min) and the amount of product was investigated using a phosphor-imaging scanner, STORM 820. The ligation efficiencies were estimated by [(amount of product)/(amount of product)+(amount of free DNA)].

F. Melting temperature analysis

Melting temperature analysis is as follow; DNA solutions (2 μM) were prepared in a phosphate buffer (1 M NaCl, 10 mM Na₂HPO₄ and 1 mM Na₂EDTA adjusted to pH 7.0 with HCl). Absorbance of solutions containing a 1:1 strand ratio of oligodeoxynucleotides at 260 nm was measured 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.

III. RESULTS

The reaction efficiencies of DNA polymerases and DNA ligases were investigated, respectively, by employing DNA substrates (Fig. 1). Both of the relative polymerization efficiencies and ligation efficiencies on the DNA substrates containing Xan:Cyt pair, Oxa:Cyt pair, or Hyp:Cyt pair (dsX:C, dsO:C, or dsH:C) were less than normal DNA duplexes (dsG:C). In addition, the efficiencies of polymerization and ligation on dsO:C were less than those of dsH:C, and the efficiencies for dsX:C were the lowest. The efficiencies of polymerization and ligation on dsX:T or dsO:T were less than those of normal DNA duplexes (dsA:T), while those of dsH:T were almost the same to those of normal dsA:T.

In general, the stability of DNA base-pairing in the catalytic site may affect on the reaction efficiencies of DNA polymerases or DNA ligases [20-28]. Thus, DNA substrates containing deaminated lesions were compared to that of normal DNA substrate in terms of their melting temperatures (*T_m*). The stability of dsH:T was almost the same as normal dsA:T, while dsX:T and dsO:T were less stable compared to normal dsA:T. Moreover, both of dsX:C and dsO:C showed low *T_m* values compared to dsG:C (even less than dsH:C). dsH:C showed *T_m* value between that of normal dsG:C and dsX:C (or dsO:C). Considering their *T_m*, the order of the stability of DNA substrates 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 found for the order of the relative reaction efficiencies of DNA ligases and DNA polymerases; dsG:C > dsH:C > dsO:C > dsX:C and dsA:T ≅ dsH:T > dsO:T > dsX:T.

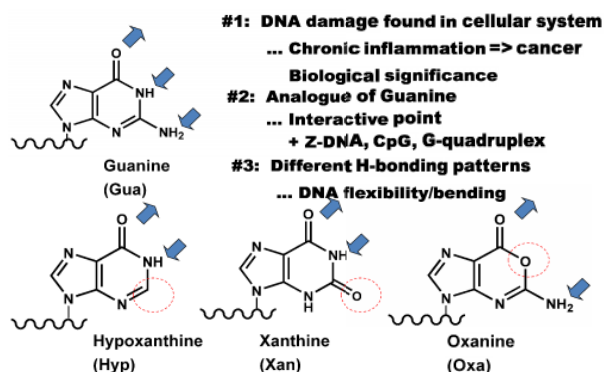


Fig. 1 Pyrimidine lesions used as bioprobe

IV. DISCUSSIONS

It is believed that the efficiencies of enzymatic reactions is strongly involved in the stability of the DNA base-pairs bound at the catalytic site of DNA polymerase and DNA ligases [20-28]. For the case of the chain elongation reaction by polymerases, such tendency was found; that is the dependency of the polymerization efficiency on the stability of the DNA substrates was observed. It has been reported that the nucleotidyl transfer reactions catalyzed by DNA polymerases are based on a two-metal-ion mechanism [22] and the fidelity of the nucleotidyl transfer reaction is involved with the energetic and structural stabilities of the base-pairs bound at the catalytic site of DNA polymerase (Fig 2A) [24, 26-27].

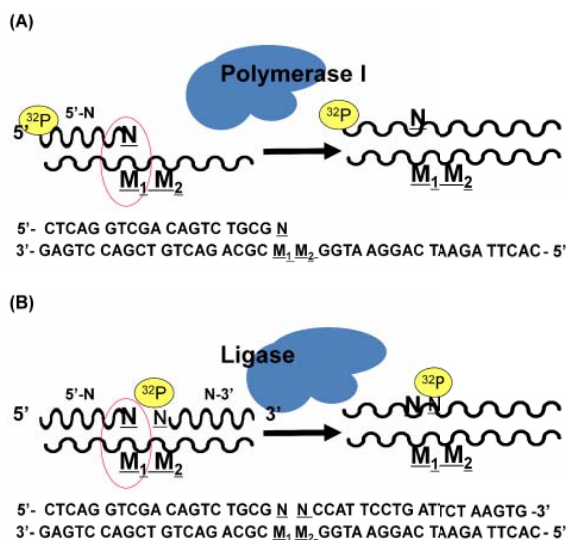


Fig. 2 Reaction mechanism of DNA polymerase (A) and DNA ligase (B)

The order of the polymerization efficiencies on containing N:C or N:T (N = G, A, X, O, H) were as follows; dsG:C > dsH:C > dsO:C > dsX:C or dsA:T \cong dsH:T > dsO:T > dsX:T, respectively. The polymerization efficiency shows the same tendency of the T_m order of the DNA substrates.

For the case of DNA ligases, similar tendency was found; DNA ligase interactively encircled the 5'-phosphorylated end of down-stream ligation fragment than the 3'-OH end of up-stream ligation fragment. Thus, the mismatched pairs at the 3'-end of up-stream ligation fragment/template might be critical for the efficiency of ligation reaction [28]. All of the base-pairs at the 5'-end of the down-stream fragment were successfully ligated even the deaminated-lesions were paired with Cyt or Thy. 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'- M_1M_2 -5'; M_1 = C or T), the efficiency of ligation was dependent on the stability between the up-stream fragment and template (Fig 2B). The order of the ligation efficiencies on DNA substrate containing N:C or N:T (N = G, A, X, O, H) was dsG:C > dsH:C > dsO:C > dsX:C or dsA:T \cong dsH:T > dsO:T > dsX:T. The ligation efficiency shows the same tendency of the T_m order of the DNA substrates.

The presence of deminated-lesions in the DNA may make negative influence on the reaction efficiencies of DNA polymerase and DNA ligase. However, the reaction efficiencies of such DNA-recognizing enzymes for deaminated-lesions in DNA duplexes were not so severely decreased. Instead, these lesions can be used for molecule-based analysis of DNA-binding/recognizing proteins or enzymes. Since the pyrimidine lesions such as Xan, Oxa and Hyp can be employed as analogues of guanine, the comparative results from their usage can provide important or new information on the role of Gua in DNA sequence in DNA-protein interaction mechanisms. This report shows such research approach to investigate molecular mechanisms of DNA-recognizing enzymes by employing such deaminated lesions as novel molecular bioprobes.

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