Two DNA Polymerases of *Escherichia coli* Display Distinct Misinsertion Specificities for 2-Hydroxy-dATP during DNA Synthesis†

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ABSTRACT: The insertion specificities of an oxidized dATP analogue, 2-hydroxydeoxyadenosine 5′-triphosphate (2-OH-dATP), were determined using the α (catalytic) subunit of *Escherichia coli* DNA polymerase III and the exonuclease-deficient Klenow fragment of DNA polymerase I. In contrast to our previous observation that mammalian DNA polymerase α incorporated the oxidized nucleotide opposite T and C, these two *E. coli* DNA polymerases incorporated 2-OH-dATP opposite T and G on the DNA template. Steady-state kinetic studies indicated that the α subunit incorporated 2-OH-dATP 10 times more frequently opposite T than opposite G. On the other hand, the incorporation of 2-OH-dATP opposite T by the exonuclease-deficient Klenow fragment was 2 orders of magnitude more efficient than that opposite G. These results indicate that the misinsertion specificity of 2-OH-dATP differs between replicative and repair-type DNA polymerases, and provide a biochemical basis for the mutations induced by 2-OH-dATP in *E. coli*.

Reactive oxygen species are generated endogenously by normal oxygen metabolism and produced by many environmental mutagens and carcinogens. For this reason, the oxidation of DNA and its precursors is believed to be a very important source of mutation and to be one of the causative factors of carcinogenesis and aging (*1*, *2*). The *mutT* mutant of *Escherichia coli*, in which A·T to C·G transversions are frequently induced (*3*), lacks the ability to hydrolyze an oxidized form of dGTP, 8-OH-dGTP[1] (*4*). Thus, damaged nucleotides appear to be important endogenous mutagens. Moreover, the presence of mammalian homologues of MutT (MTH1 proteins) supports this speculation (*5*).

When we treated dA and dATP with reactive oxygen species-generating reagents, 2-hydroxy-dA and 2-OH-dATP, respectively, were produced as efficiently as 8-hydroxy-dG-(TP) (*6*, *7*). It has been demonstrated that 2-OH-dATP was more mutagenic than 8-OH-dGTP when both were directly introduced into *E. coli* cells (*8*). Furthermore, we recently found that the human MTH1 protein hydrolyzes 2-OH-dATP more efficiently than 8-OH-dGTP (*9*). These findings suggest that this oxidized form of dATP, 2-OH-dATP, is also an important endogenous mutagen. Thus, it is of great interest to examine the miscoding properties of 2-OH-dATP during DNA synthesis.

We previously reported that mammalian DNA pol α incorporated 2-OH-dATP opposite T and C on a template DNA in vitro (*6*). This result suggests that the spectrum of mutations caused by 2-OH-dATP in cells only involves G·C → A·T transitions. However, 2-OH-dATP induced G·C → T·A transversions in the chromosomal *lacI* gene when the oxidized nucleotide was introduced directly into *E. coli* cells, suggesting misincorporation of 2-OH-dATP opposite G (*8*). Thus, we raised the question of whether *E. coli* DNA polymerases could misincorporate 2-OH-dATP opposite G in contrast to mammalian pol α.

We report here that the catalytic subunit of *E. coli* DNA polymerase III and *E. coli* KF^{exo-} misincorporated 2-OH-dATP opposite G on the DNA template.

EXPERIMENTAL PROCEDURES

Materials. T4 polynucleotide kinase was purchased from Toyobo. The α subunit of *E. coli* DNA pol III was purified according to the method described previously (*10*). The exonuclease-deficient Klenow fragment of DNA polymerase I (KF^{exo-}) was from New England Biolabs. The FPLC-grade deoxynucleoside triphosphates used in the DNA polymerase reactions were from Amersham Pharmacia Biotech. 2-OH-dATP was prepared by treatment of dATP with Fe(II)-EDTA-O_2 and was purified by HPLC as described previously (*6*). The purified nucleotide was eluted as a single peak during both reverse-phase and anion-exchange HPLC (data not shown). The nucleotide purified by this procedure was

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[1] Abbreviations: 2-OH-dATP, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate; 2-OH-Ade, 2-hydroxyadenine; 8-OH-dGTP, 8-hydroxy-2′-deoxyguanosine 5′-triphosphate; pol III, DNA polymerase III; pol α, DNA polymerase α; KF^{exo-}, exonuclease-deficient Klenow fragment of DNA polymerase I; KF^{exo+}, exonuclease-proficient Klenow fragment of DNA polymerase I; F_{ins}, frequency of insertion.

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Table 1: Oligonucleotide Sequences

sequences[a]	oligonucleotides
5'-dCCAAAACGTCG-3'	Primer-1 (11mer)
3'-dCGTCGGTTTTGCAGC**N**GCTTAAGAT-5'	Template-1 (25mer)
5'-dACCACCAGCT-3'	Primer-2 (10mer)
3'-dTGGTGGTCGA**N**TAT-5'	Template-2 (14mer)

[a] **N** = A, G, C, or T.

eluted at the same time as authentic 2-hydroxy-dA during reverse-phase HPLC after dephosphorylation (*6*). 2-OH-dATP was stable under the conditions of the DNA polymerase reactions described below (data not shown). Purified oligonucleotides were from Hokkaido System Science (Sapporo, Japan).

In Vitro DNA Synthesis. Primer DNA (10 pmol) was 5′-end labeled by T4 polynucleotide kinase (10 units) with [γ-^{33}P]ATP (0.74 MBq, NEN Life Science Products) in a reaction mixture (10 μL) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. After incubation at 37 °C for 60 min, the unincorporated ATP was removed with NENSORB 20 (NEN Life Science Products).

Reactions catalyzed by the α subunit were carried out in a reaction mixture containing the primed oligonucleotide template (0.05 μM), 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 8 mM dithiothreitol, 2% glycerol, 80 μg/mL bovine serum albumin, various concentrations of dNTP, and the enzyme at 30 °C. Experiments with KF^{exo-} were conducted in a reaction mixture containing the primed oligonucleotide template (0.05 μM), 50 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 5 mM 2-mercaptoethanol, various concentrations of dNTP, and the enzyme at 20 °C.

Reactions were stopped by the addition of a termination solution (95% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol). Samples were heated at 95 °C for 3 min and were then applied to a 7 M urea, 20% polyacrylamide gel. Autoradiograms were obtained with a Fujix BAS 2000 Bio Image Analyzer.

The Michaelis constant (K_m) and the maximum velocity of the reaction (V_{max}) were obtained from Lineweaver−Burk plots of the kinetic data (*11*). Insertion frequencies (F_{ins}) were determined relative to T·dATP or G·dCTP according to the equations developed by Mendelman et al. (*12*). To obtain steady-state kinetic parameters, reactions catalyzed by the α subunit were carried out at 30 °C for 1−10 min. Reactions catalyzed by KF^{exo-} were carried out at 20 °C for 6 min.

RESULTS

Incorporation of 2-OH-dATP by the E. coli DNA Polymerase III Catalytic Subunit. Using DNA templates with defined sequences, we analyzed the incorporation of 2-OH-dATP during in vitro DNA synthesis catalyzed by the α subunit of *E. coli* DNA pol III. Template 1 (25mer) annealed to ^{33}P-labeled primer 1 (11mer) was used in the reactions (Table 1). Figure 1A shows the pattern of primer extension catalyzed by the α subunit on different DNA templates. The primer annealed to template 1T (N = T, Table 1) was elongated by the α subunit to give a 12mer after the addition of 2-OH-dATP (Figure 1A, lane 5). Moreover, the incorporation of 2-OH-dATP was observed even when primer 1 was annealed to template 1G (N = G, Table 1) (lane 3). These

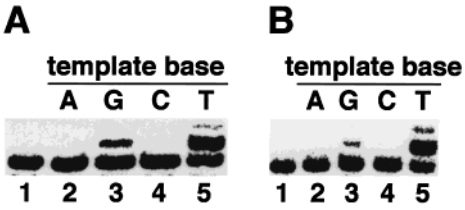

FIGURE 1: Incorporation of 2-OH-dATP by the α subunit of DNA polymerase III (A) and the exonuclease-deficient Klenow fragment (B). (A) Template 1 was annealed to ^{33}P-labeled primer 1. The template−primer complex (0.05 μM) was treated with the α subunit (40 units) in the presence of 50 μM 2-OH-dATP under the conditions described in Experimental Procedures. The reaction mixtures were incubated at 30 °C for 60 min and were processed as described in Experimental Procedures. (B) The template−primer complex (0.05 μM) was treated with KF^{exo-} (0.02 unit) in the presence of 50 μM 2-OH-dATP under the conditions described in Experimental Procedures. The reaction mixtures were incubated at 20 °C for 30 min and were processed as described in Experimental Procedures: lane 1, untreated primer 1; lane 2, template 1A; lane 3, template 1G; lane 4, template 1C; and lane 5, template 1T.

results indicate that the α subunit could incorporate 2-OH-dATP opposite T and G. The incorporation of 2-OH-dATP opposite T was preferential to that opposite G. When template 1T was used, further elongation from a 12mer to a 13mer was detected (lane 5). This also indicates that 2-OH-dATP was incorporated opposite the G located immediately 5′ of the targeted T (see Table 1). We arrived at the same conclusion when another primed template (primer 2 and template 2, Table 1) was used (data not shown).

Incorporation of 2-OH-dATP by the Exonuclease-Deficient Klenow Fragment of E. coli DNA Polymerase I. We next analyzed in vitro DNA synthesis by KF^{exo-} in the presence of 2-OH-dATP. As shown in Figure 1B, the results were similar to those obtained with the α subunit. 2-OH-dATP was incorporated opposite T and G, and incorporation opposite T was favored over that opposite G (Figure 1B, lanes 3 and 5). Thus, the two *E. coli* DNA polymerases were able to insert 2-OH-dATP opposite G.

Steady-State Kinetics. We measured the kinetic parameters during the in vitro DNA synthesis. The incorporation of 2-OH-dATP opposite T and G was examined using template 1 (Table 1). The results of kinetic studies are shown in Figure 2 and Table 2.

In reaction mixtures containing the α subunit and template 1T, the K_m value of 2-OH-dATP was 20 times higher than that of dATP. The relative V_{max} value of the incorporation of 2-OH-dATP was 3.5 times lower than that of dATP. The relative V_{max}/K_m (the F_{ins}) of 2-OH-dATP was 1.4×10^{-2}, which means that the insertion of 2-OH-dATP opposite T was disfavored by a factor of 70 compared to that of dATP. With the α subunit and template 1G, the K_m value of 2-OH-dATP was 13 times higher than that of dCTP. The relative V_{max} value of the incorporation of 2-OH-dATP was 60 times lower than that of dCTP. The relative V_{max}/K_m (the F_{ins}) of 2-OH-dATP was 1.2×10^{-3}, which means that the insertion of 2-OH-dATP opposite G was 800 times less frequent than that of dCTP. When the incorporation of 2-OH-dATP opposite T and that opposite G were compared, the K_m value and the relative V_{max} value were 5.9 and 60 times lower, respectively, in the case of G·2-OH-dATP pair formation (Table 2). Thus, the insertion of 2-OH-dATP opposite T was favored only 10-fold over that opposite G. These results are

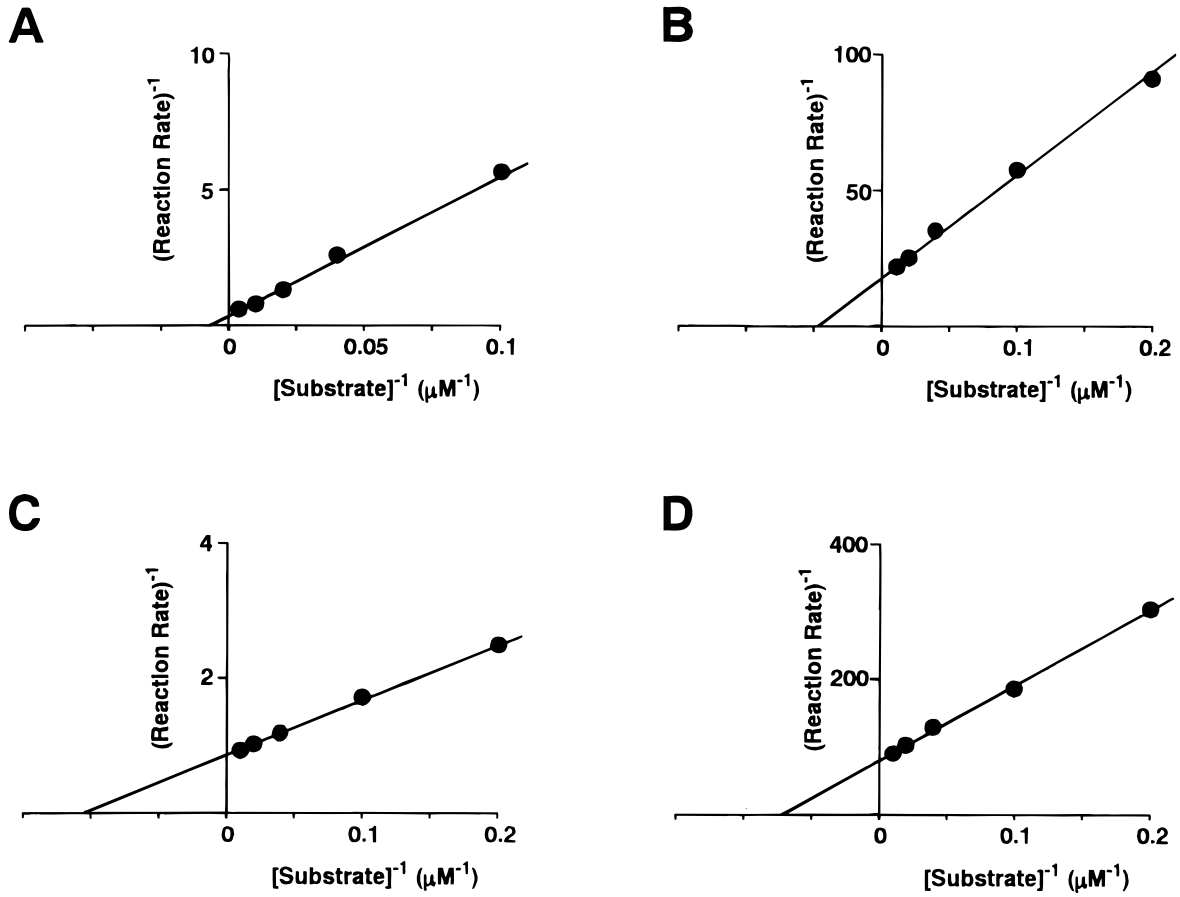

FIGURE 2: Lineweaver−Burk plots of 2-OH-dATP insertion reactions conducted by the α subunit (A and B) and by KF^{exo-} (C and D). Template 1T (A and C) or template 1G (B and D) was used.

Table 2: Kinetic Parameters of Nucleotide Insertion Reactions by DNA Polymerases

DNA polymerase	template base·dNTP	K_m (μM)	relative V_{max}	V_{max}/K_m (μM^{-1})	F_{ins}
α subunit of pol III	T·dATP	5.8	10.8	1.9	1.0
	T·2-OH-dATP	118	3.1	0.026	1.4×10^{-2}
	G·dCTP	1.5	3.1	2.1	1.0
	G·2-OH-dATP	20	0.052	0.0026	1.2×10^{-3}
KF^{exo-}	T·dATP	0.070	1.0	14	1.0
	T·2-OH-dATP	9.3	1.4	0.15	1.1×10^{-2}
	G·dCTP	0.097	1.8	19	1.0
	G·2-OH-dATP	13	0.011	0.00085	4.5×10^{-5}

in agreement with our observation that 2-OH-dATP is mutagenic and elicits G·C → T·A transversions when introduced into *E. coli* (*8*). The F_{ins} of 2-OH-dATP opposite G was much higher than that of dATP opposite G (1.2×10^{-5}) reported in the literature (*13*).

In reaction mixtures containing KF^{exo-} and template 1T, the K_m value of 2-OH-dATP was 130 times higher than that of dATP. The relative V_{max} value of the incorporation of 2-OH-dATP was 1.4 times higher than that of dATP. The finding that the V_{max} value of the incorporation of 2-OH-dATP was higher than that of unmodified dATP evokes the result obtained with pol α (*6*). The relative V_{max}/K_m (the F_{ins}) of 2-OH-dATP was 1.1×10^{-2}, which means that the insertion of 2-OH-dATP opposite T was disfavored by a factor of 90 compared to that of dATP. With KF^{exo-} and template 1G, the K_m value of 2-OH-dATP was 130 times higher than that of dCTP. The relative V_{max} value of the incorporation of 2-OH-dATP was 160 times lower than that of dCTP. The relative V_{max}/K_m (the F_{ins}) of 2-OH-dATP was 4.5×10^{-5}, which means that the insertion of 2-OH-dATP opposite G was disfavored by a factor of 20 000 compared to that of dCTP. When the incorporation of 2-OH-dATP opposite T and that opposite G were compared, the K_m value was 1.4 times higher and the relative V_{max} value was 130 times lower in the case of G·2-OH-dATP pair formation (Table 2). Thus, the incorporation of 2-OH-dATP opposite T was favored by about 200 times over that opposite G.

DISCUSSION

An oxidized form of dATP, 2-OH-dATP, was previously found to induce G·C → T·A transversions frequently in a chromosomal gene in *E. coli* (*8*). This in vivo result is supported by our present findings which show that the α subunit of *E. coli* DNA pol III prefers to incorporate 2-OH-dATP opposite T rather than opposite G by a factor of 10 (Table 2). Therefore, the high mutagenicity of oxidized dATP could be due to the high misincorporation rate of the *E. coli* replicative DNA polymerase.

The ability of the α subunit to discriminate between T·2-OH-dATP and T·dATP mainly relies on its different K_m

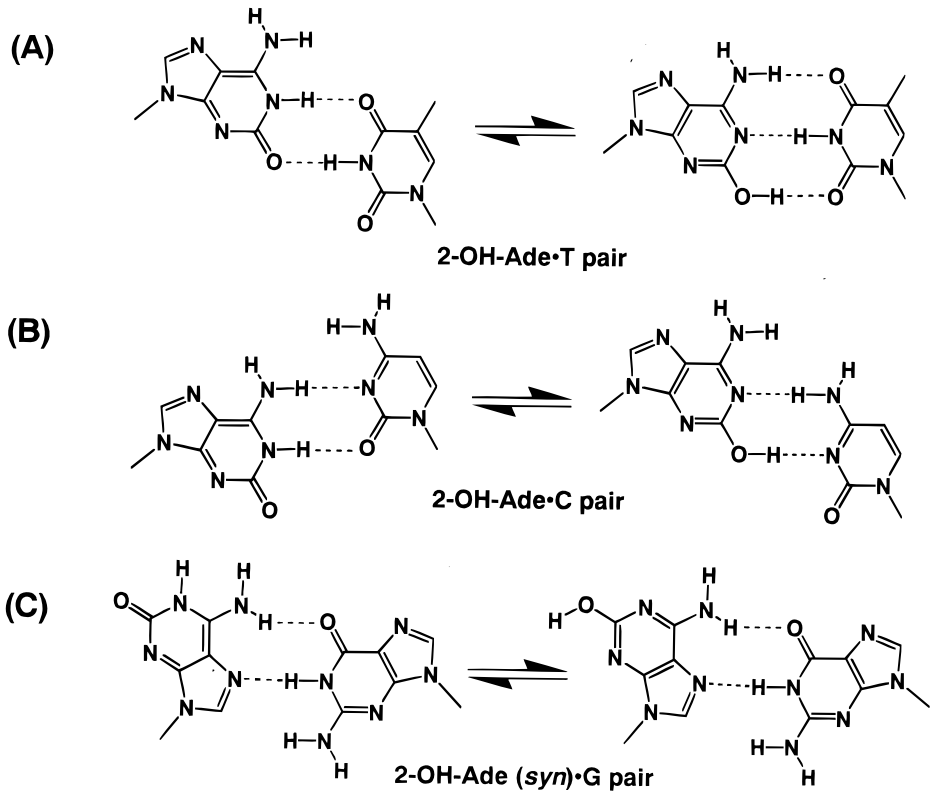

FIGURE 3: Postulated base pairs involving 2-OH-Ade: (A) 2-OH-Ade•T pair, (B) 2-OH-Ade•C pair, and (C) 2-OH-Ade(syn)•G pair.

values for these substrates (Table 2). The V_{max} values differed by only 3.5-fold. On the other hand, the ability of the α subunit to discriminate against G•2-OH-dATP and G•dCTP relies on both the higher K_m and lower V_{max} values**.** Similar results were obtained with KF^{exo-}. This enzyme discriminated against T•2-OH-dATP and T•dATP at the level of the K_m and against G•2-OH-dATP and G•dCTP through the higher K_m and lower V_{max} values (Table 2).

We observed that the V_{max} values for the incorporation of 2-OH-dATP opposite T were 3.5 times lower and 1.4 times higher than those for the incorporation of dATP opposite T with the α subunit and KF^{exo-}, respectively (Table 2). Interestingly, mammalian pol α inserts 2-OH-dATP opposite T with a 1.5-fold higher V_{max} than for dATP (*6*). This may be due to the presence of a 2-enol (2-hydroxy) tautomer during T•2-OH-dATP base pairing, which would involve a three-hydrogen bond base pair with T and yield a geometry similar to that of Watson−Crick type base pairing (Figure 3A). This putative base pair may be more stable than the A•T base pair and may facilitate rapid phophodiester bond formation. Recent structural analysis suggests the formation of this base pair in DNA (*14*).

Previously, we did not find misincorporation of 2-OH-dATP opposite G during in vitro DNA synthesis with KF^{exo+} (*6*), although KF^{exo-} inserted 2-OH-dATP opposite G (Figure 1B). This discrepancy suggests that the proofreading activity of this polymerase removes 2-hydroxy-dAMP residues opposite G. It was reported that the exonuclease activities of DNA polymerases reduce the frequency of A → C mutations induced by 8-OH-dGTP (*15*). Thus, 8-hydroxy-dGMP residues misincorporated opposite A appear to be substrates for the exonuclease activities of DNA polymerases. The proofreading function of DNA polymerases is expected to suppress the high level of mutagenesis that can be caused by these damaged nucleotides in vivo. Moreover, the relative V_{max} value for the incorporation of 2-OH-dATP opposite T was 1.4-fold higher than that for dATP with KF^{exo-} (Table 2). However, the relative V_{max} value for the incorporation of 2-OH-dATP opposite T was 8 times lower than that for dATP when KF^{exo+} was used (*6*). The difference in the F_{ins} values (1.1×10^{-2} vs 1.3×10^{-3}, Table 3) reflects this V_{max} effect. Although sequence contexts were different in the two studies, these results suggest that T•2-OH-dATP, in addition to G•2-OH-dATP, is a substrate for the exonuclease activity of this polymerase.

We recently found that 2-OH-dATP, present in the reaction mixture containing gapped heteroduplex and the *E. coli* DNA polymerase III holoenzyme, frequently induces G → T transversions (*16*). This result indicates that the extension from 2-OH-dAMP at the 3′-end (opposite G) was conducted by the DNA polymerase III holoenzyme that contains the exonuclease activity.

Similar kinetic studies have been reported for 8-OH-dGTP (*4*, *17*−*19*). Table 3 summarizes F_{ins} values of 2-OH-dATP and 8-OH-dGTP obtained by in vitro DNA synthesis experiments. The F_{ins} values of 2-OH-dATP are comparable to those of 8-OH-dGTP. Therefore, DNA polymerases seem to utilize 2-OH-dATP as efficiently as 8-OH-dGTP. In particular, pol α appears to incorporate 2-OH-dATP more efficiently than 8-OH-dGTP, although the F_{ins} value of 8-OH-dGTP opposite A has not been reported. The α subunit of pol III misincorporates 8-OH-dGTP opposite A 1.3-fold more frequently than opposite C (Table 3). Thus, misinsertion of 8-OH-dGTP occurs more frequently than misinsertion of 2-OH-dATP. However, 2-OH-dATP induced substitution mutations in the *lacI* gene more frequently than 8-OH-dGTP when these nucleotides were introduced directly into *E. coli* (*8*). Many factors other than misincorporation, such as

Table 3: Insertion of Oxidized Nucleotides by Various DNA Polymerases[a]

DNA polymerase	template base·2-OH-dATP	F_{ins}	template base·8-OH-dGTP	F_{ins}
α subunit of pol III	T·2-OH-dATP	1.4×10^{-2}	C·8-OH-dGTP	2.9×10^{-2}
	G·2-OH-dATP	1.2×10^{-3}	A·8-OH-dGTP	3.9×10^{-2}
KF^{exo-}	T·2-OH-dATP	1.1×10^{-2}	C·8-OH-dGTP	4.4×10^{-4}
	G·2-OH-dATP	4.5×10^{-5}	A·8-OH-dGTP	7.2×10^{-4}
			C·8-OH-dGTP	6.5×10^{-6}
			A·8-OH-dGTP	2.8×10^{-6}
KF^{exo+}	T·2-OH-dATP	1.3×10^{-3}	−	−
pol α	T·2-OH-dATP	3.4×10^{-3}	C·8-OH-dGTP	8.0×10^{-6}
	C·2-OH-dATP	7.5×10^{-4}	A·8-OH-dGTP	NA[b]

[a] Data for 2-OH-dATP are taken from Table 2 (the α subunit and KF^{exo-}) and from ref *6* (KF^{exo+} and pol α). Data for 8-OH-dGTP are collected from refs *4* and *17*−*19*. [b] Not available.

hydrolysis by the MutT or MutT type enzymes, the removal of misincorporated nucleotides by the exonuclease activities of DNA polymerases, and DNA repair, affect the level of mutagenesis that can be caused by damaged nucleotides. Further studies will be necessary to explain the mutagenicity of 2-OH-dATP observed in vivo.

We have shown that two *E. coli* DNA polymerases, the α subunit and KF^{exo-}, inserted 2-OH-dATP opposite G and T (Figure 1). On the other hand, mammalian DNA pol α inserts 2-OH-dATP opposite C and T (*6*). Thus, the misincorporation mode of 2-OH-dATP is DNA polymerase-specific. Interestingly, misincorporation of nucleotides opposite 2-OH-Ade in DNA templates is also DNA polymerase-specific (*18*, *19*). 2-OH-Ade·dGTP and 2-OH-Ade·dCTP pairs were formed by KF^{exo-} and mammalian DNA polymerases, respectively. Moreover, induction of an A → C transversion by 2-OH-Ade occurs in *E. coli* (*22*) but not in mammalian cells (*23*). Thus, the base pairing properties of 2-OH-Ade during DNA synthesis, either in DNA or in a monomeric form, depend on the DNA polymerases that are involved.

The reason the mispairing properties of 2-OH-Ade depend on the nature of the DNA polymerase remains to be determined. One possibility is that the hydrophobicity of the active sites of DNA polymerases might affect the enol−keto equilibrium of 2-OH-Ade (*24*, *25*). This putative equilibrium shift would have an important effect on the formation of a base pair involving 2-OH-Ade. Another possibility is that the "shape" of the active site determines the efficiency of base pair formation involving 2-OH-dATP. When 2-OH-dATP is incorporated opposite C, wobble base pairs may be formed (Figure 3B). On the other hand, various base pairs are possible in the case of G·2-OH-dATP. For example, 2-OH-dATP may adopt the syn conformation and may pair with G in the template DNA (Figure 3C). If the putative pair were accepted by the α subunit but not by mammalian pol α due to interactions with amino acid residues at the active site, the characteristic incorporation of 2-OH-dATP opposite G by the α subunit could be explained. Similar speculation about other putative base pairs involving 2-OH-dATP can be entertained.

In this study, we found that the two *E. coli* DNA polymerases could incorporate 2-OH-dATP opposite G. This result is in contrast to our previous finding which showed that mammalian DNA pol α inserts 2-OH-dATP opposite C and T in DNA. This result indicates that the misincorporation mode of 2-OH-dATP depends on the nature of the DNA polymerase. To our knowledge, this is the only damaged nucleotide for which the misinsertion specificity has been determined, although some DNA lesions exhibit different miscoding properties depending on the DNA polymerase (*26*−*28*). The results shown here provide a biochemical basis for the G·C → T·A transversions induced by 2-OH-dATP in *E. coli* (*8*).

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