Translesion Synthesis across Bulky N²-Alkyl Guanine DNA Adducts by Human DNA Polymerase κ*

Received for publication, March 9, 2006, and in revised form, April 6, 2006. Published, JBC Papers in Press, June 1, 2006, DOI 10.1074/jbc.M602246200

Jeong-Yun Choi†‡, Karen C. Angel†, and F. Peter Guengerich†‡

From the †Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146 and the ‡Department of Pharmacology, College of Medicine, Ewha Womans University, 911-1 Mok-6-Dong, Yangcheon-Gu, Seoul 158-710, Republic of Korea

DNA polymerase (pol) κ is one of the so-called translesion polymerases involved in replication past DNA lesions. Bypass events have been studied with a number of chemical modifications with human pol κ, and the conclusion has been presented, based on limited quantitative data, that the enzyme is ineffective at incorporating opposite DNA damage but proficient at extending beyond bases paired with the damage. Purified recombinant full-length human pol κ was studied with a series of eight N²-guanyl adducts (in oligonucleotides) ranging in size from methyl- to -CH₂(6-benzo[α]pyrenyl) (BP). Steady-state kinetic parameters (catalytic specificity, $k_{cat}/K_m$) were similar for insertion of dCTP opposite the lesions and for extension beyond the N²-adduct G:C pairs. Mispairing of dGTP and dTTP was similar and occurred with $k_{cat}/K_m$ values $\sim 10^{-3}$ less than for dCTP with all adducts; a similar differential was found for extension beyond a paired adduct. Pre-steady-state kinetic analysis showed moderately rapid burst kinetics for dCTP incorporations, even opposite the bulky methyl(9-anthracenyl)- and BPG adducts ($k_p 5.9 \sim 10.3 \text{s}^{-1}$). The rapid bursts were abolished opposite BPG when α-thio-dCTP was used instead of dCTP, implying rate-limiting phosphodiester bond formation. Comparisons are made with similar studies done with human pols η and λ; pol κ is the most resistant to N²-bulk and the most quantitatively efficient of these in catalyzing dCTP incorporation opposite bulky guanine N²-adducts, particularly the largest (N²-BPG).

DNA is constantly damaged by chemical and physical agents. When the damage is not repaired it can contribute to genomic instability and probably to several maladies, including cancer, cardiovascular disease, and aging (1, 2). Basic issues involved in the understanding of the etiology of mutation include the biochemical formation of electrophiles and oxygen radicals, characterization of their interactions with DNA, the enzymatic repair of damage, and the processing of DNA to generate permanent changes in the genetic information. In this latter regard, the enzymology of DNA replication is of considerable interest.

The mutation of DNA cannot be understood only in the context of preferred base pairing, in that the free energy differences among various pairs of natural and modified DNA bases are not sufficient to explain normal or abnormal pairing (3). Thus, the major driving forces in the generation of mutations from damaged DNA are the polymerases (4). The normal replicative DNA polymerases are the first that encounter DNA adducts, and some small DNA adducts (e.g. O⁶-MeG and 8-oxo-7,8-di-hydroG)² are processed by these polymerases, at least part of the time. However, a group of DNA polymerases first discovered in the late 1990s participates in “translesion” synthesis beyond small and large DNA adducts, although the process is, in general, less efficient and more error-prone than the reactions that replicative polymerases catalyzed with unmodified DNA (1, 5).

One of these “Y-family” translesion polymerases is polymerase (pol) κ, studied in mammals. pol κ was discovered as a homolog of the Escherichia coli dinB gene product (pol IV), an enzyme involved in the SOS adaptive response (6, 7). The crystal structure of a core element of human pol κ has been determined (8). The ability of pol κ to bypass a number of types of DNA damage has been reported, including guanine modified with N-acetylaminofluorene or BPDE (9–14), xanthine (15), 8-nitroguanine (16), and guanine modified with oxidized estrogens (17). However, pol κ has not been judged to be very efficient in bypassing UV photoproducts (9, 18), cis-platin-modified DNA (10), 1,N⁶-ethenodeoxycytidine (19), O⁶-MeG (20), or 8-(hydroxymethyl)-3,N⁴-ethenoC (21). Other issues involving pol κ include up-regulation in lung cancer (22), a possible (but not established) role in somatic hypermutation (23), and a general role in genome maintenance versus instability (24).

A case has been made that pol κ acts mainly as an “extender” of damaged DNA that has already undergone insertions opposite modified bases, as opposed to a role in misinsertion itself (20, 25, 26). Few of the studies on pol κ provide very much

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* This work was supported by United States Public Health Services Grants R01 ES10375 and P30 ES00267 (to F. P. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, 638 Robinson Research Bldg., 23rd and Pierce Avenues, Nashville, TN 37232-0146. Tel: 615-322-2261; Fax: 615-322-3141; E-mail: f.guengerich@vanderbilt.edu.

8 The abbreviations used are: Me, methyl; Et, ethyl; Ib, isobutyl; Bz, benzyl; N²-Naph, N²-Ch₂(2-naphthyl); N²-Anth, N²-Ch₂(9-anthracenyl); N²-BP, N²-Ch₂(6-benzo[a]pyrenyl); BPDE, benzo[a]pyrene diol epoxide; dCTPakers, durerase; RT, reverse transcriptase; T7, bac-to phage DNA polymerase T7, exonuclease-deficient; HIV-1, human immunodeficiency virus, type 1.
Effects of N²-Guanine Adducts on DNA pol κ

quantitative information about catalysis of either misinsertion as further extension. Some steady-state kinetic studies have been done, but many only report steady-state rates as percentages and cannot be converted to $k_{cat}$ values. Also, after applying some assumptions about the actual enzyme concentrations used, many of the rates appear to be very low. Even if valid steady-state kinetic analyses are reported, their usefulness is limited by the complexity of the polymerase catalytic cycle (27).

To obtain a better perspective on the role of human pol κ, we prepared a purified full-length enzyme and utilized this in kinetic and other studies with a defined oligonucleotide containing a G or each of eight N²-substituted Gs at the same site. Thus, the system varies only in the size of the lesion (with associated changes in hydrophobicity and electronic properties), and the results obtained with pol κ can be compared directly with corresponding studies done with HIV-1 RT and bacteria-ophage T7 (-28) and two other human Y-family polymerases, pol η (29) and pol λ (30). Our results indicate that pol κ is particularly resilient to blockage with the larger adducts, as judged by several criteria, and that incorporation opposite the particular lesion is limited by the complexity of the polymerase catalytic cycle (27).

**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled dNTPs were obtained from Amer sham Biosciences, (S₂)-dCTPαS was from Biolog Life Science Institute (Bremen, Germany), and [γ-³²P]ATP (specific activity 3 × 10⁸ Ci mmol⁻¹) was from PerkinElmer Life Sciences. T₄ polynucleotide kinase and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Bio-spin columns were obtained from Bio-Rad. A protease inhibitor mixture (mixture) was from Roche Applied Science (Indianapolis, IN). Human testis cDNA was purchased from BD Biosciences Pharmingen (San Diego, CA). Amicon Ultra centrifugal filter devices were purchased from Millipore (Billerica, MA).

**Oligonucleotides**—Unmodified 24-mer, 25-mers, and 36-mer (Table 1) were purchased from Midland Certified Reagent Co. (Midland, TX). Eight 36-mers, each containing a guanine N²-adduct (N²-MeG, N²-EtG, N²,N²-diMeG, N²-IbG, N²-BzG, N²-NaphG, N²-AnthG, and N²-PBG) were prepared as previously described and characterized by capillary gel electrophoresis and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (28–30). The extinction coefficients for the oligonucleotides, estimated by the Borer method (31) were as follows: 24-mer, $\varepsilon_{260} = 224$ mm⁻¹ cm⁻¹; 25-mer, $\varepsilon_{260} = 232$ mm⁻¹ cm⁻¹; and 36-mer, $\varepsilon_{260} = 310$ mm⁻¹ cm⁻¹ (28, 29).

**Isolation of Human POLK cDNA and Construction of Recombinant Baculovirus**—Human DNA polymerase κ cDNA was obtained by PCR amplification from human testis cDNA (as template) using Pfu ultra DNA polymerase with the corresponding two primers 5′-CTCGAGGGCATGGATAGCA CAAAGGA-3′ and 5′-GGATTATTGCACTTGGCTTCA-3′. The resulting 2.7-kb PCR product of POLK was cloned into the vector pCR-Script Amp, and nucleotide sequencing was used to confirm the entire sequence of the coding region. The fragment containing the human POLK gene was cloned into the vector pAcHLT-B using the Xhol and NotI sites to generate a fusion tag containing six histidines at the N terminus, yielding the vector pAcHLT/HPOLK. The plasmid was co-transfected into SF9 insect cells using BaculoGold DNA (with a BaculoGold transfection kit) to generate the recombinant baculovirus expressing human pol κ.

**Expression and Purification of Human DNA Polymerases**—Recombinant human pol κ P3 viral stocks were used to infect 600-ml spinner flask cultures of SF9 cells at 1 × 10⁶ cells ml⁻¹. The infected cells were incubated for 60 h and harvested by centrifugation for 10 min at 4 °C at 3 × 10⁶ × g. Cells were resuspended in 50 ml of Buffer A (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol) containing 0.5% (v/v) Nonidet P-40 and Roche Complete protease inhibitor mixture (one tablet per 50 ml). Cell debris was pelleted by ultracentrifugation for 60 min at 4 °C at 105,000 × g. The resulting supernatant was loaded (0.3 ml min⁻¹) onto a 5-ml HisTrap HP affinity column (Amersham Biosciences) in an Amersham Biosciences Äkta fast-protein liquid chromatography system at 4 °C. The column was washed (at 0.8 ml min⁻¹) with 50 ml of Buffer A containing 20 mM imidazole, 50 ml of Buffer A containing 40 mM imidazole, and then with 50 ml of Buffer A containing 50 mM imidazole. Bound enzyme was eluted with 400 mM imidazole (in buffer A). Fractions containing pol κ were collected, diluted 5-fold with Buffer B (50 mM Tris-HCl (pH 7.5), 10% glycerol (v/v), 5 mM 2-mercaptoethanol, and 1 mM EDTA), and loaded onto a 1-ml Mono S column (Amersham Biosciences). Unbound protein was washed from the column with 20 ml of Buffer B containing 350 mM NaCl, and pol κ was eluted with a 30-ml linear gradient of 0.35 to 2 mM NaCl in Buffer B. Eluted fractions (0.26 ml) were analyzed by SDS-polyacrylamide gel electrophoresis with silver staining (32). pol κ was found to be eluted at 600 mM NaCl. Pooled fractions were concentrated using an Amicon Ultra centrifugal filter (Millipore) to a volume of 100 μl and further purified to near homogeneity using a Superdex 200 column (Amersham Biosciences) with Buffer B containing 150 mM NaCl. Fractions containing >95% pure recombinant protein were pooled, concentrated, and exchanged into storage buffer (50 mM Tris-HCl, pH 7.5, 50% glycerol (v/v), 1 mM dithiothreitol, and 1 mM EDTA). The active enzyme concentration of pol κ was determined by the active site titration method (see below).

Recombinant human pol δ was prepared as described previously (29). Recombinant human PCNA was prepared in E. coli and purified as described (33, 34).

**Reaction Conditions for Enzyme Assays**—Standard DNA polymerase reactions were done in 50 mM Tris-HCl (pH 7.5) buffer containing 5 mM dithiothreitol, 100 μg bovine serum albumin ml⁻¹ (w/v), and 10% glycerol (v/v) with 100 mM primer-template at 37 °C (28–30). Primers were 5′-end-labeled using T4 polynucleotide kinase/[γ-³²P]ATP and annealed with template (36-mer). All reactions were initiated by the addition of dNTP solutions containing MgCl₂ (5 mM final concentration) to preincubated enzyme/DNA mixtures.

**Primer Extension Assay with All Four dNTPs (“Run-on” or “Starting Point Experiments”)—A ³²P-labeled primer, annealed to either an unmodified or modified (N²-alkylG) template, was
Effects of N2-Guanine Adducts on DNA pol κ

extended in the presence of all four dNTPs (100 μM each) for 15 min. Reaction mixtures (8 μl) were quenched with 2 volumes of a solution of 20 mM EDTA in 95% formamide (v/v) (28). Products were resolved using a 16% polyacrylamide (w/v) gel electrophoresis system containing 8 M urea and visualized using a Bio-Rad Molecular Imager FX and Quantity One software (Bio-Rad).

Steady-state Kinetic Analyses—A 32P-labeled primer, annealed to either an unmodified or adducted template, was extended in the presence of varying concentrations of a single dNTP. The molar ratio of primer/template complex to enzyme was at least 10:1. Polymerase concentrations and reaction times were chosen so that maximal product formation was ≥20% of the substrate concentration (35). The primer-template was extended with dNTP in the presence of 0.05–10 mM pol κ for 5 or 10 min. All reactions (8 μl) were done at ten dNTP concentrations (in duplicate) and quenched with 2 volumes of a solution of 20 mM EDTA in 95% formamide (v/v) (28–30). Products were resolved using a 16% polyacrylamide (w/v) electrophoresis gel containing 8 M urea and quantitated by phosphorimaging analysis using a Bio-Rad Molecular Imager FX instrument and Quantity One software. Graphs of product formation versus dNTP concentration were fit using nonlinear regression (hyperbolic fits) in GraphPad Prism Version 3.0 (San Diego, CA) for the determination of Kcat and Km values.

Pre-steady-state Reactions—Rapid quench experiments were performed using a model RQF-3 KinTek Quench Flow Apparatus (Kin’Tek Corp., Austin, TX). Reactions were initiated by rapid mixing of 32P-primer/template/pol κ mixtures (12.5 μl) with the dNTP-Mg2+ complex (10.9 μl) and then quenched with 0.3 mM EDTA after times varying from 10 ms to 2 s (or 4 s for N3-BPG-containing DNA). Reaction products were mixed with 450 μl of formamide-dye solution (20 mM EDTA, 95% formamide (v/v), 0.5% bromphenol blue (w/v), and 0.05% xylene cyanol (w/v)) and separated using a denaturing electrophoresis gel, with quantitation as described for the steady-state reactions. Pre-steady-state experiments were fit with the burst equation

\[
y = A(1 - e^{-k_{tr}t}) + k_{nt}t,\]  

where y = concentration of product, A = burst amplitude, ktr = pre-steady-state rate of nucleotide incorporation, t = time, and knt = steady-state rate of nucleotide incorporation (not normalized for enzyme concentration in the equation) (36, 37), using nonlinear regression analysis in GraphPad Prism Version 3.0.

Phosphorothioate Analysis—With the 32P-primer annealed to either an unmodified or adducted template, reactions were initiated by rapid mixing of 32P-primer/template/pol κ mixtures (12.5 μl) with (S)DTP:SaMg2+ complex (or dCTP:Mg2+ (10.9 μl) and then quenched with 0.3 mM EDTA after reaction times varying from 10 ms to 2 s (or 4 s for N3-BPG-containing DNA). Products were analyzed as described for the pre-steady-state reactions (see above).

Active Site Titration and Determination of KdDNA—The Kd (DNA) for productive binding of pol κ to 24-mer/36-mer DNA substrates was determined using pre-steady-state analysis, pol κ (preincubated with increasing concentrations of the DNA substrate in sample syringe A) was mixed with a saturating concentration of dCTP:Mg2+, from sample syringe B, and then quenched with 0.3 mM EDTA after a time interval of 0.2 s. A graph of the burst amplitude versus total DNA concentration was plotted and fit to a quadratic equation,

\[
A = 0.5(K_D + E_n + D) - \left[0.25(K_D + E_n + D)^2 - E_n D\right]^{1/2},
\]

where A = burst amplitude, E = active enzyme concentration, D = DNA concentration, and Kd = equilibrium dissociation constant for productive DNA binding (36, 37) in GraphPad Prism Version 3.0.

Table 1

| Oligodeoxynucleotides used in this study |
|----------------------------------------|
| 24-mer                                 |
| 25C-mer                                |
| 25T-mer                                |
| 36-mer                                 |

*G = G, N2-MeG, N2,EtG, N2,N2-diMeG, N2-IeG, N2-BzG, N2-NaphG, N2-AntG, or N3-BPG.

**FIGURE 1.** N2-Guanine derivatives used with pol κ.

RESULTS

Overall Strategy—The goal of the research was to characterize the interactions of pol κ with damaged DNA and compare these with other human translesion DNA polymerases, particularly pols η and λ. Full-length human pol κ was prepared (see “Experimental Procedures” and Supplemental Fig. S1) and used in a series of quantitative steady-state and pre-steady-state kinetic measurements with a set of oligonucleotides in which a single G was substituted at the N2 atom with a series of adducts varying only in size (Table 1 and Fig. 1). Thus, the only variable involved in this comparison is the adduct size (although the electronic properties and hydrophobicity (30) of alkyl and aryl groups should not be ignored, in the context of base stacking (4)). Similar studies have been done with the replicative DNA polymerases HIV-1 RT and bacteriophage pol T7 (exo−) (28) and human pol η (29) and pol λ (30), and comparison of the properties of these three major human translesion DNA polymerases is now possible.

Primer Extension by Human pol κ Using All Four dNTPs—The first set of studies involved extension of the primer in the presence of all four dNTPs when paired with a template containing either a G or an N2-alkylG (fixed incubation time and...
Effects of N²-Guanine Adducts on DNA pol κ

FIGURE 2. Extension of 32P-labeled primers opposite G, N²-MeG, N²-EtG, N²-IbG, N²-BzG, N²-NaphG, N²-AnthG, and N²-BPG by human pol κ with all four dNTPs present. A primer (24-mer) was annealed with each of the eight different 36-mer templates (Table 1) containing an unmodified G or N²-modified G placed at the 25th position from the 3'-end. Reactions were done for 15 min with increasing concentrations of pol κ (0–8 nM) and 100 nM DNA substrate (primer/template) as indicated. The 32P-labeled 24-mer primer was extended in the presence of all four dNTPs. Reaction products were resolved by denaturing gel electrophoresis with subsequent phosphorimaging analysis.

TABLE 2

Steady-state kinetic parameters for one-base incorporation by human pol κ

| Template | dNTP | Km μM | kcat s⁻¹ | kcat/Km | Misinsertion ratio |
|----------|------|--------|-----------|---------|-------------------|
| G        | C    | 8.4 ± 0.9 | 0.31 ± 0.03 | 37 | 1.0008 |
|          | G    | 1300 ± 300 | 0.036 ± 0.003 | 0.028 | 0.0015 |
|          | T    | 6800 ± 700 | 0.38 ± 0.02 | 0.056 | 0.0015 |
| N²-MeG   | C    | 11 ± 0.7 | 0.13 ± 0.01 | 12 | 0.0053 |
|          | G    | 700 ± 60 | 0.044 ± 0.001 | 0.063 | 0.0053 |
|          | T    | 6800 ± 1000 | 0.29 ± 0.03 | 0.048 | 0.0041 |
| N²-EtG   | C    | 22 ± 2 | 0.36 ± 0.01 | 16 | 1.0000 |
|          | G    | 860 ± 160 | 0.023 ± 0.002 | 0.027 | 0.0016 |
|          | T    | 1600 ± 660 | 0.15 ± 0.03 | 0.094 | 0.0057 |
| N²-IbG   | C    | 11 ± 1 | 0.66 ± 0.01 | 60 | 1.0000 |
|          | G    | 330 ± 60 | 0.027 ± 0.001 | 0.082 | 0.0014 |
|          | T    | 1600 ± 600 | 0.17 ± 0.04 | 0.11 | 0.0018 |
| N²-BzG   | C    | 6.2 ± 0.5 | 0.38 ± 0.01 | 61 | 1.0000 |
|          | G    | 220 ± 20 | 0.12 ± 0.001 | 0.55 | 0.0090 |
|          | T    | 410 ± 80 | 0.045 ± 0.003 | 0.11 | 0.0018 |
| N²-NaphG | C    | 11 ± 1 | 0.27 ± 0.01 | 25 | 1.0000 |
|          | G    | 460 ± 90 | 0.087 ± 0.006 | 0.19 | 0.0078 |
|          | T    | 870 ± 140 | 0.17 ± 0.01 | 0.20 | 0.0082 |
| N²-AnthG | C    | 47 ± 9 | 0.24 ± 0.01 | 5.1 | 1.0000 |
|          | G    | 320 ± 60 | 0.0017 ± 0.0001 | 0.0053 | 0.0010 |
|          | T    | 890 ± 160 | 0.0080 ± 0.0007 | 0.0089 | 0.0017 |
| N²-BPG   | C    | 32 ± 3 | 0.045 ± 0.001 | 1.4 | 0.0038 |
|          | G    | 390 ± 80 | 0.0021 ± 0.0001 | 0.0053 | 0.0038 |
|          | T    | 760 ± 120 | 0.0063 ± 0.0004 | 0.0082 | 0.0059 |

varying concentrations of pol κ) (Fig. 2). Although PCNA has been reported to stimulate pol κ activity (38), it was only apparent in the presence of other accessory factors, i.e. replication factor C and replication protein A, with long and end-closed DNA templates (e.g. M13 circular template or 75-mer DNA templates) (29) and much lower than we have measured under similar conditions for several replicative DNA polymerases (28, 34–39) but similar to those for Sulfolobus solfataricus Dpo4 (44, 45) and human pol η (29) and much lower than for human pol ι (30). The misinsertion ratios were low for pol κ, even with the bulkiest adducts, where the proclivity to misinsert dGTP and dTTP was similar to that seen for G itself.

Steady-state Kinetics of dNTP Incorporation by pol κ Opposite G and N²-G Adducts—Preliminary analyses with single dNTPs indicated that dCTP, dTTP, and dGTP were inserted opposite all of the N²-alkylG adducts and dATP was not. Steady-state kinetic analyses were done with varying concentrations of dCTP, dTTP, and dGTP under conditions that favor only the incorporation of a single base and the oligomer substrate is not depleted (Table 2). One possibility that cannot be dismissed, in the absence of sequence analysis of larger products, is that the (limited) dGTP insertion occurs opposite the C base 5' to the adduct (Table 1).

Several points are in order. The Km values for dCTP, the correct nucleotide, are higher with pol κ than we have measured under similar conditions for several replicative DNA polymerases (28, 34–39) but similar to those for Sulfolobus solfataricus Dpo4 (44, 45) and human pol η (29) and much lower than for human pol ι (30). The misinsertion ratios were low for pol κ, even with the bulkiest adducts, where the proclivity to misinsert dGTP and dTTP was similar to that seen for G itself.
Another important point is that $k_{\text{cat}}/K_m$, the catalytic specificity, only decreased by one order of magnitude when going from G to the very bulky N2-BPG.

Steady-state Kinetics of Next-base Extension Following G and N2-G Adducts Paired with C or T—The literature reports a number of accounts of extension of pol $\kappa$ past adducts paired with inserted bases, and the view has been presented that this function is more facile than insertion (26, 46, 47). Steady-state kinetic analysis was done with either C or T positioned opposite G or the N2-alkylG adduct (Table 3).

The kinetic parameters were quantitatively similar to those measured for insertion reactions (Table 2). A strong tendency to extend the G/N2-alkylG:C pairs was observed, relative to pairs with T. As in the case of insertion opposite 2-alkylG adduct (Table 3).

Pre-steady-state Burst Kinetics of dCTP Incorporation Opposite G, N2-AnthG, and N2-BPG by pol $\kappa$—Steady-state kinetic parameters are useful in understanding DNA polymerases but are limited in the amount of information they can provide in terms of mechanistic details about catalysis (27). To our knowledge, pre-steady-state kinetic measurements have not been reported previously with pol $\kappa$, and analyses were done to characterize aspects of catalysis (Fig. 3).

For dCTP incorporation opposite G, clear burst kinetics was observed (Fig. 3). The shape of the burst is not as sharp as in the case of several of the highly replicative polymerases we have studied (34, 40, 45) and in human pol $\eta$ (29). The “break” between the first cycle and the linear, steady-state phase is less pronounced and reminiscent of the patterns seen with $E. coli$ pol I (Klenow fragment, exonuclease−) and pol II (exonuclease−) (39) (but more defined than seen with pol $\kappa$ (30)). The patterns can be understood largely in the context of the rate of forward progress through the catalytic cycle relative to rates of release of the DNA substrate/product from the enzyme (4, 30).

The burst rates are modestly rapid, even with a bulky adduct present (N2-AnthG) (Fig. 3). Even with the very bulky N2-BPG adduct, a weak burst was observed. The observation of partial bursts with a system for the same oligonucleotide (as a function of having an adduct present) is evidence for the existence of an inactive polymerase-DNA (dNTP) complex, in equilibrium with the active form (48, 49).

The existence of a burst indicates that the slow step in steady-state catalysis occurs after formation of the product, e.g. product release (50). This is clearly the case for dCTP insertion opposite G and N2-AnthG. With dCTP incorporation opposite N2-BPG, both the burst rate and the steady-state rates are attenuated. We hypothesize that this phenomenon is the result of increased rates of incorporation, not DNA release from the enzyme. We did not measure $k_{\text{off}}$ rates in this work with pol $\kappa$, but in our experience with all other DNA polymerases we have not observed major changes in $k_{\text{off}}$ or $k_0$ due to DNA modification (4).
Effects of N²-Guanine Adducts on DNA pol κ

**FIGURE 4.** Phosphorothioate analysis of pre-steady-state kinetics of nucleotide incorporation by human pol κ. pol κ (17 nM) was incubated with 100 nM 24-mer/36-mer primer-template complexes (32P-labeled primer) in a rapid quench-flow instrument and mixed with 1 mM dCTP (▷) or (S₃)dCTPαS (▼) to initiate reactions for the 24-mer/36-G-mer (A), 24-mer/36-N²-Anth-mer (B), and 24-mer/36-N²-BPG-mer (C). Pre-steady-state rates (kₚ) were determined from the burst equation (Fig. 3), and solid lines represent the best fits. The following rates were estimated: A, dCTP, kₚ = 10.7 ± 3.2 s⁻¹; (S₃)dCTPαS, kₚ = 3.3 ± 0.9 s⁻¹; B, dCTP, kₚ = 10.3 ± 4.3 s⁻¹; (S₃)dCTPαS, kₚ = 1.4 ± 0.6 s⁻¹; C, dCTP, kₚ = 5.9 ± 1.0 s⁻¹; (S₃)dCTPαS, no burst (linear rate, k = 0.05 ± 0.01 s⁻¹).

**FIGURE 5.** Determination of KₖDNA for human pol κ by active site titration. pol κ (final nominal concentration, 120 nM) was incubated with varying 24-mer/36-G-mer (32P-labeled) concentrations (6–150 nM) and mixed with 1 mM dCTP to initiate the reaction. Reactions were quenched with EDTA after 0.2 s, and product formation was analyzed by gel electrophoresis and phosphorimaging. A plot of burst amplitudes versus total DNA concentration was fit to a quadratic equation (see "Experimental Procedures"), yielding an active site concentration of 21.1 ± 0.9 nM and KₖDNA = 11.5 ± 2.3 nM.

Phosphorothioate Analysis of dCTP Incorporation Opposite G, N²-AnthG, and N²-BPG Adducts by pol κ—One approach to further delineation of aspects of catalysis by DNA polymerases is the use of sulfur/oxygen elemental effects on rates. The principle is similar to that utilized with kinetic isotope effects, in that the substitution of an α-oxygen of a dNTP with sulfur decreases the electronegativity and makes bond breakage more difficult. If this is a slow step in the reaction, then the overall reaction will be slowed (51, 52). This is a simplistic interpretation and, as with many aspects of kinetic hydrogen isotope effects, the detailed interpretation of the extent of the changes has some differences in interpretation (53).

When these studies were done with pol κ, only a relatively small difference in burst rates was observed in incorporation of dCTP opposite G (Fig. 4). The difference (the thio effect) was much greater in the case of dCTP incorporation opposite N²-AnthG and N²-BPG (Fig. 4). For the respective two adducts, the thio effects were 7 and ~120.

Active Site Titration and Estimation of the Productive KₖDNA for pol κ—The observation of burst kinetics (Figs. 3 and 4) allowed for the estimation of several parameters using kinetic means. One analysis is the "active site" titration (Fig. 5). In this experiment, the Kₖ for DNA was estimated to be 12 nM (Fig. 5). This value is similar to that estimated for pol η (29) and most of the replicative DNA polymerases (34, 39, 40) but ~5-fold less than for pol ε (30) or, interestingly, bovine pol δ (34).

Determination of KₖdCTP (dCTP Incorporation) by pol κ—The initial analyses of pre-steady-state kinetics (Fig. 3) provide estimates of the rate of burst phase (kₚ), but this requires extrapolation to high concentration to estimate kₚpol. These experiments were done (Fig. 6). The kₚpol values for dCTP incorporation opposite G and the bulky adduct N²-AnthG were identical, and the KₖdCTP value for incorporation opposite N²-AnthG was ~2-fold higher than opposite G.

The KₖdNTP values are much higher than reported for processive DNA polymerases, i.e. 1–4 μM (34), but are similar to those reported for other translesion DNA polymerases (29, 30, 54). The adduct does not make a large difference in the KₖdNTP. Another point of interest is that the KₖdNTP values are much higher than the Kₘ values reported for these reactions (Table 2), and the specific contribution of Kₖ to the parameter Kₘ is not clear.

Primer Extension (All Four dNTPs) Opposite N²-EtG and N²,N²-diMeG by Human pol δ and κ—pol δ appears to utilize Hoogsteen base pairing instead of (or at least in addition to) the classic Watson-Crick mode (55, 56). One way of analyzing the ability of polymerases to “replace” Watson-Crick hydrogen bonding with a Hoogsteen mode is to compare the abilities to insert N²,N²-diMeG relative to N²-EtG (30), because these two lesions are nearly isosteric, but N²,N²-diMeG eliminates the N2 hydrogen atom from a Watson-Crick pairing mode. When
Effects of N²-Guanine Adducts on DNA pol κ

these experiments were done in another setting (30), human pol η did not show activity with N²,N²-diMeG, but human pol λ did (30), consistent with the proposal that Hoogsteen base pairing is operative with pol λ (55, 56).

When these experiments were done with pol δ, little incorporation opposite N²,N²-diMeG was observed (Fig. 7A). With pol κ, some incorporation opposite N²,N²-diMeG and extension were observed (Fig. 7B), indicating that pol κ may have some limited capability to utilize non-Watson-Crick pairing modes.

The system was evaluated in more detail, using steady-state kinetic analysis (Table 4). The $k_{cat}/K_m$ for incorporation of dCTP opposite N²,N²-diMeG was 47-fold lower than for N²-EtG with pol κ, compared with a 2,000-fold difference with pol δ/PCNA. The major misincorporation for pol δ was dATP and that for pol κ was dTTP. The misincorporation showed less attenuation due to removal of the N2 hydrogen. These results are consistent with the view of a finite but limited role for non-Watson-Crick pairing with pol κ.

FIGURE 6. Estimation of $K_{cat}^{dCTP}$ for human pol κ by pre-steady-state burst rate dependence on dCTP concentration. pol κ (17 nM) was incubated with 100 nM 24-mer/36-mer primer-template complex (32P-labeled) in a rapid quench-flow instrument and mixed with varying dCTP concentrations (50–1000 μM) to initiate reactions. Reactions were quenched with EDTA, and product formation was analyzed following electrophoresis and phosphorimaging. A plot of burst rates ($k_{cat}^{dCTP}$ versus dCTP) was fit to a hyperbolic equation (see “Experimental Procedures”). A, G (unmodified): $k_{cat}^{dCTP} = 190 ± 30$ nM, $k_{cat} = 13 ± 1$ s⁻¹ and $K_{cat}^{dCTP} = 190 ± 30$ μM; B, N²-AnthG: $k_{cat} = 13.8 ± 0.6$ s⁻¹ and $K_{cat}^{dCTP} = 340 ± 30$ μM.

FIGURE 7. Extension of 32P-labeled 24-mer primer complexed with templates containing N²-EtG and N²,N²-diMeG by human pol δ and κ. The 32P-labeled 24-mer primer was annealed with 36-mer templates (Table 1) containing an N²-EtG or N²,N²-diMeG placed at the 25th position from the 3′-end. The 32P-labeled 24-mer primer was extended in the presence of all four dNTPs for 15 min with varying amounts of pol δ (0–20 nM, with excess PCNA (34)) and pol κ (0–4 nM) and a constant concentration of DNA substrate (100 nM primer-template), as indicated. The reaction products were analyzed by denaturing gel electrophoresis with subsequent phosphorimaging. A, pol δ/PCNA; B, pol κ.

DISCUSSION

A series of kinetic studies were done with purified recombinant human pol κ and a defined set of oligonucleotides varying only in the size of a substitution at the guanine N2 atom, with the only changes being in the associated steric bulk, hydrophobicity, and electronic properties. Although the view has been advanced that the role of pol is to extend primers past a base incorporated opposite an adduct, the quantitative evidence to support this view is very limited. We have used a number of more quantitative approaches to address this issue, at least within the context of the set of N²-G modifications. The results indicate that pol κ is relatively proficient in inserting dCTP opposite the modified guanines as well as extending, pairs, as demonstrated using steady-state and pre-steady-state approaches. Although the catalytic specificity constant, $k_{cat}/K_m$, has some deficiencies in describing enzyme reactions, it is a useful first approximation of enzyme efficiency and provides a basis for understanding polymerase function as a function of molecular size of adducts. Such plots for incorporation (dCTP, dTTP, and dGTP) (Fig. 8A) and extension beyond N²-G adducts (paired with C or T) (Fig. 8B) can be used as the basis for several conclusions: (i) pol κ is much more efficient than (human) pol η or pol λ in incorporating dCTP opposite large N²-G adducts (N²-AnthG and N²-BPG) (29, 30); (ii) the preference for insertion of dCTP relative to other dNTPs is similar to that of pol η (and better with the bulkier adducts) (29) and much better than that of pol λ (30); (iii) pol κ is much less influenced by adduct size than any other DNA polymerases studied in similar systems (28–30); and (iv) similar conclusions can be drawn about extension beyond N²-G adducts:C pairs (Fig. 8B). The conclusions about insertion are reinforced by the more qualitative results of the “run-on” experiments (Fig. 2) and the burst kinetics observed in the pre-steady-state analyses (Figs. 3 and 4). Overall, these studies provide a better understanding of the action of pol κ, at least with N²-alkyl- and -aralkylG adducts.

Although the view has been advanced that the role of pol κ is to extend primers from a base incorporated opposite an adduct by other polymerases, the quantitative evidence to support this view is very limited in that it has been tested only with some adducts such as 3′T of the T-T dimer and O²-MeG (20, 25), and...
Effects of N²-Guanine Adducts on DNA pol κ

TABLE 4

Comparison of steady-state kinetic parameters for one-base incorporation opposite N²-EtG and N²,N²-diMeG by human pol κ and δ

| Polymerase | Template-dNTP | Kₘ | kcat | kcat/Km | Difference of kcat/Km compared to N²-EtG | f |
|------------|---------------|-----|------|--------|----------------------------------------|---|
| Pol κ      | N²-EtG-dCTP   | 22 ± 2 | 0.36 ± 0.01 | 16.4 | 1 | 1 |
|           | N²,N²-diMeG-dCTP | 780 ± 100 | 0.27 ± 0.01 | 0.35 | 47-fold lower | 1 |
|           | N²-EtG-dTTP    | 1600 ± 600 | 0.15 ± 0.03 | 0.094 | 0.0057 |
|           | N²,N²-diMeG-dTTP | 2000 ± 200 | 0.14 ± 0.01 | 0.07 | No significant difference | 0.2 |
|           | N²-EtG-dCTP    | 3.5 ± 0.6 | 0.014 ± 0.001 | 4.0 | 1 |
|           | N²,N²-diMeG-dCTP | 1400 ± 600 | 0.0029 ± 0.0006 | 0.002 | 2000-fold lower | 1 |
|           | N²-EtG-dATP    | 610 ± 80 | 0.012 ± 0.001 | 0.019 | 0.0048 |
|           | N²,N²-diMeG-dATP | 360 ± 60 | 0.0018 ± 0.0001 | 0.005 | 4-fold lower | 2.5 |

FIGURE 8. Effect of the volume of adduct at the guanine N2 atom on catalytic efficiency (kcat/Km) for dCTP incorporation opposite N²-G adducts by human pol κ. A, molecular volumes (Å³) of adducts at the guanine N2 atom were calculated using the program Chem3D (version 7.0), based on the Connolly surface algorithm (57), and plotted against kcat/Km values (Table 2) for dCTP ( ), dATP ( ), and dTTP ( ) incorporation for various N²-G adducts by pol κ. B, molecular volumes (Å³) of adducts at the guanine N2 atom are plotted against kcat/Km values (Table 3) for next-base extension (with dGTP) from matched G (or N²-G adducts)/C template-primer termini ( ) or mismatched G (or N²-G adducts)/T template-primer termini ( ) by pol κ.

no direct comparisons have been made for extension ability with other translesion polymerases. The kcat/Km values for next-base extension can be compared with those for base incorporation opposite various N²-alkylG adducts by each polymerase (Supplemental Fig. S2), and the plots clearly indicate that pol κ is almost as efficient in incorporation opposite lesion as in extension. pol δ is rather slightly more efficient in extension than incorporation. Interestingly, pol κ is less efficient in extension than incorporation with the mismatched G:T base pair, but the opposite pattern is observed with the matched G:C base pair.

The comparisons of the abilities of pol κ to insert opposite (and copy past) N²-EtG and N²,N²-diMeG (Fig. 7 and Table 4) provide some insight into the mode of binding of nucleotide triphosphates. Moderate loss of efficiency and fidelity opposite N²-EtG indicates that the error-free bypass opposite N²-G adducts (at least N²-EtG) by pol κ may depend considerably on the Watson-Crick pairing, in agreement with a recent report (58). However, the dependence on the third hydrogen bonding capability during bypass by pol κ is much less than for pol δ and η (30), and pol κ exhibited some activity toward N²,N²-diMeG, at least more than human pol δ/PCNA. Binding of dCTP opposite N²,N²-diMeG does not involve classic Watson-Crick pairing, because no N2 hydrogen is available (at least with this atom). pol γ, for which structural evidence exists to support a Hoogsteen pairing mode (55, 56), is proficient in insertion opposite N²,N²-diMeG (30). Our present results suggest that pol κ has some limited, but not extensive, ability to utilize non-Watson-Crick modes of pairing or other possible supportive interactions, which might be specialized for N²-bulk itself in the minor groove side. A crystal structure of the core of human pol κ has been published (8) but without DNA. Models have been made with DNA inserted into the available structure, based on the available structures of yeast pol η and S. solfatarius Dpo4 (8), although the conclusions cannot support or refute the possibility of non-Watson-Crick pairing for pol κ. We also note a recent report that a steric gate residue (Phe) has a critical role in bypass of some N²-G adducts by E. coli DinB (DNA polymerase IV), an apparent orthologue of human pol κ (59).

The available results support the general DNA polymerase mechanism shown in Fig. 9. Some support for the contribution of a non-productive polymerase-DNA (dNTP) complex is provided by the partial bursts seen with the N²-G adducts in Fig. 3 (48), although further experiments are to prove this. In a typical steady-state experiment with excess oligonucleotide (e.g. Table 2), a step following product formation must be rate-limiting, at least for incorporation opposite G and the less bulky adducts, because of the burst kinetics (Fig. 3). Although we did not measure kcat rates for pol κ, we hypothesize that step 7 (of Fig. 9) is probably rate-limiting and has a major influence on kcat, as with most DNA polymerases (27, 37). Within the catalytic cycle itself
Effects of N²-Guanine Adducts on DNA pol κ

**FIGURE 9. General kinetic mechanism for DNA polymerization.** Individual steps are numbered and discussed in the text. E = pol (K), Dₜ = DNA substrate, E⁺ = conformationally altered polymerase, E⁻ = nonproductive conformation of polymerase, Dₚₓ₋₁ = DNA extended by one base, and PP₁ = pyrophosphate.

(steps 2-6 of Fig. 9), either step 3 (a proposed conformational change) or 4 (phosphodiester bond formation) is rate-limiting. Step 3 has precedent in studies with other DNA polymerases, although its identity is controversial (60, 61). Some key experiments to establish the existence of step 3 have not been done with pol κ, e.g. pulse-chase versus pulse-quinch (60, 62). The elemental influence on rates (i.e. thio effects, Fig. 4) are not without controversy regarding their interpretation (53, 61, 63, 64). However, the results are quite clear (Fig. 4), and the patterns resemble those observed for several other DNA polymerases, i.e. low thio effects equal to normal incorporation and high thio effects equal to the rate of misincorporation or incorporation of the "right" dNTP opposite an adduct (39, 40, 65, 66). The most direct interpretation of such results, such as these, is that a "non-chemical" step is rate-limiting for normal base incorporation (step 3 of Fig. 9) but, as the reaction becomes more difficult, then step 4 becomes limited. Such an interpretation has been challenged on the basis that the sulfur substitution can change steric influences as well as electronic (53, 61, 63, 64).

The data sets available for these N²-G adducts (28-30) can be summarized and compared. The replicative polymerases (T7⁻, HIV-1 RT, and human pol δ/PCNA) are very efficient in normal incorporation but very sensitive to even a small substitution at the N2 atom of G. The incorporation that does occur with large adducts is with dATP. Human pol δ is actually more tolerant of size than the two viral polymerases (HIV-1 RT and T7⁻). The Y-family translesion polymerases are less efficient but more tolerant of the adduct size (or other associated properties, i.e. hydrophobicity and electronic effects). pol η shows the highest fidelity with N²-G adducts up to N²-NaphG (i.e. inserting dCTP opposite) but loses the fidelity with larger adducts, whereas pol κ has the highest fidelity throughout the whole series (Fig. 8A). pol κ was clearly the most tolerant of the larger adducts.

Finally, the available information about N²-G adducts can be considered in a more general view with what happens in mammalian systems, based on the work now available with human pols δ, η, λ, and κ (29, 30). With small N²-G lesions (Me or Et), pol δ can bypass these in an error-free manner (30). With larger lesions, pol δ stalls and one of the translesion polymerases is more effective. With an N²-G lesion smaller than N²-NaphG, any of these three polymerases can catalyze bypass replication. pol λ is error-prone (30) and pols η and κ are relatively error-free. With adducts as large as or larger than N²-AnthG, pol η or κ is active, the former being error-prone (29) and the latter being relatively error-free (Fig. 8). The base sequence context can be a major factor for error-free or error-prone bypass in this step, in that pol κ shows 3 orders of magnitude of differences in Vₘₐₓ (kₐₚ)/Kₘₜ of dCTP incorporation opposite (+)-trans-anti-benzo[a]pyrene diol epoxide-modified-N²-G in different sequences (14), although having no available information for pol η. The possible role of pol κ in efficient and error-free bypass of N²-BPDE-modified-G has also been suggested by quantitative analysis in mammalian cells (68). Because the rates of polymerization are not competitive with dissociation, the translesion polymerases have distributive behavior and leave the DNA, being replaced soon by pol δ.

These studies have a reductionist character by their very nature, and caution needs to be used in extrapolation to other adducts. However, we are of the opinion that systematic studies of this type are necessary to understand catalysis by these polymerases, in that the battery of DNA adducts already studied (with pol κ (9-21)) varies considerably in positional, steric, and electronic properties and that any quantitative conclusions are
difficult. Finally, the systems are undoubtedly more complex in cells. Still other DNA polymerases are present, and the contributions of accessory proteins cannot be ignored. In this regard, we have utilized PCNA with pol δ (30, 34) but have uniformly found no stimulation of activity, in studies with short oligonucleotides, with human pols η, ι, and κ (29, 30) (see above), and Gerlach et al. (10) also reported a lack of effect of PCNA on pol κ. However, the situation with large pieces of DNA and in vivo may differ, and specific translational modifications of PCNA (ubiquitination and sumoylation) have been investigated for their effects (69–71), although doing quantitative in vitro investigations with such systems remains a challenge.

In summary, the enzymatic properties of the recombinant human translesion DNA polymerase pol κ have been studied. Although the amounts of the full-length protein available were very limited, it was possible to carry out a number of the most relevant steady-state and pre-steady-state experiments because of the high sensitivity available using 32P-labeled substrates. Studies with a set of defined N2-G adducts varying only in size were utilized, and the quantitative results allow the conclusion that pol κ catalyzes relatively efficient and high fidelity incorporation of dCTP opposite these adducts, being rather refractory to bulk size. Insertion and extension beyond a pair proceed with similar efficiency. The contributions of different steps in the catalytic cycle of pol κ changes with the size of the N2-G lesion, and some involvement of non-Watson-Crick base pairing of dCTP with the N2-G adducts may occur. Finally, we conclude that pol κ may be considered to be the most efficient and accurate enzyme for bypass of bulky N2-guanine minor groove DNA adducts, at least among those considered here.

Acknowledgment—We thank K. Trisler for assistance in preparation of the manuscript.

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Effects of N2-Guanine Adducts on DNA pol κ

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Translesion Synthesis across Bulky N²-Alkyl Guanine DNA Adducts by Human DNA Polymerase κ
Jeong-Yun Choi, Karen C. Angel and F. Peter Guengerich

J. Biol. Chem. 2006, 281:21062-21072.
doi: 10.1074/jbc.M602246200 originally published online June 1, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M602246200

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