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Effect of *N*²-Guanyl Modifications on Early Steps in Catalysis of Polymerization by *Sulfolobus solfataricus* P2 DNA Polymerase Dpo4 T239W

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Summary

Translesion DNA polymerases are more efficient at bypass of many DNA adducts than replicative polymerases. Previous work with the translesion polymerase *Sulfolobus solfataricus* Dpo4 showed a decrease in catalytic efficiency during bypass of bulky *N*²-alkyl deoxyguanosine (G) adducts with *N*²-isobutylG showing the largest effect, decreasing ~120-fold relative to unmodified dG (Zhang, H. *et al.* (2009) *J. Biol. Chem.* **284**, 3563-3576). The effect of adduct size upon individual catalytic steps has not been easy to decipher because of the difficulty of distinguishing early non-covalent steps from phosphodiester bond formation. We developed a mutant with a single Trp (T239W) to monitor fluorescence changes associated with a conformational change that occurs after binding a correct dNTP (Beckman, J.W. *et al.* (2008) *J. Biol.* **283**, 36711-36723) and, in the present work, utilized this approach to monitor insertion opposite *N*²-alkylG-modified oligonucleotides. We estimated maximal rates for the forward conformational step, which coupled with measured rates of product formation yielded rate constants for the conformational step (both directions) during insertion opposite several *N*²-alkylG adducts. With the smaller *N*²-alkylG adducts, the conformational rate constants were not changed dramatically (< 3-fold), indicating that the more sensitive steps are phosphodiester bond formation and partitioning into inactive complexes. With the larger adducts (≥ (2-naphthyl)methyl) the absence of fluorescence changes suggests impaired ability to undergo an appropriate conformational change, consistent with previous structural work.

Keywords

DNA polymerase; DNA adducts; translesion DNA synthesis; fluorescence; pre-steady-state kinetics

Introduction

The preservation and transmittal of accurate genetic information is dependent upon the fidelity of DNA replication.¹ The role of Watson-Crick base pairing in storing genetic information has

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been recognized since the elucidation of the structure of DNA,² but energetic considerations argue that the specificity of G:C and A:T pairing is not sufficient to account for the selectivity and the accuracy of the genetic code is instead maintained to a large extent by DNA polymerases through various mechanisms.³

Most DNA polymerases catalyze dNTP incorporation according to a general polymerization mechanism (Fig. 1). First, enzyme and DNA bind to form a binary complex, which then binds dNTP to form a polymerase-DNA-dNTP ternary complex and induces a conformational change to facilitate the formation of a phosphodiester bond. After the reaction, PP_i is released and the ternary complex is relaxed to initiate a new cycle. The crystal structures of ternary complexes have revealed large nucleotide-induced conformational changes for some DNA polymerases.⁴⁻¹⁰ Efficient polymerization results from a conformational change that appropriately aligns the primer 3'-hydroxyl and the α -phosphate group of the incoming dNTP for phosphodiester bond formation. However, in at least one instance (pol β) the large "closing" event observed in the crystal structure was shown to be too fast to be significantly rate-limiting in the kinetic mechanism,^{11,12} and a more subtle change is considered to be more relevant to discrimination by the DNA polymerases.¹³ This conformational change step prior to phosphoryl transfer has been considered to be very significant in nucleotide selection and polymerase fidelity.¹³⁻¹⁵

Although crystal structures are important they are static, and dynamic fluorescence approaches can provide kinetic information about conformational changes in a reaction pathway. Several methods have been used with DNA polymerases. 2-AP can be positioned at the site of incorporation or the 5'-next template position and has been used to study conformational changes with *Escherichia coli* pol I and bacteriophage pol T4,^{16,17} pol β ,¹⁸⁻²⁰ RB69,^{21,22} and *Sulfolobus acidocaldarius* Dbh,²³ with the fluorescence signal dependent on dNTP-induced protein structure changes. The contribution of local conformational changes in the enzyme structure to the changes observed with 2-AP is not entirely clear.^{21,24,25} Stopped-flow fluorescence resonance energy transfer methods have been used with *E. coli* DNA pol I^{25,26} and Klentaq1²⁷⁻²⁹ by attaching a donor and an acceptor at different positions of the protein or DNA to report changes in protein structure or DNA movement. Covalent attachment of an environmentally sensitive dye to the finger domain has also been used to monitor conformational changes with bacteriophage T7 DNA polymerase (pol T7-),^{30,31} although the question of whether a local conformational change near the dye or a global movement of the finger subdomain is involved has been raised.²⁵ Single-molecule and ensemble fluorescence measurements³² as well as hydrogen-deuterium exchange methods³³ have also been used to study conformational changes in DNA polymerases.

Recently our group reported that *Sulfolobus solfataricus* Dpo4 T239W and N188W have similar activity to wild-type Dpo4 and can be directly used to monitor conformational changes that occur during DNA polymerization, without the use of dyes and without altering the catalytic efficiency of the enzyme.³⁴ Previous kinetic work with Dpo4 has shown that this model Y-family DNA polymerase shows decreased catalytic efficiency during bypass of *N*²-alkylG adducts that is dependent on the increasing size of the moiety attached to the N2 atom of guanine.³⁵ The availability of kinetic and structural data related to Dpo4-catalyzed bypass of *N*²-alkylG adducts provides an excellent opportunity to use kinetic monitoring of conformational changes to understand the effect of bulk upon individual steps in the polymerase catalytic cycle. In order to discriminate between the effects of DNA adducts bulk on conformational change and phosphodiester bond formation, early steps in the incorporation of dCTP opposite *N*²-alkylG adducts were studied using Dpo4 T239W.

Results

Steady-state kinetics of dCTP incorporation opposite G and *N*²-alkylG adducts by Dpo4 T239W

A 13-mer primer/18-mer template duplex (Table 1) containing G or various *N*²-alkylG adducts (*N*²-MeG, *N*²-IbG, *N*²-BzG, *N*²-NaphG, *N*²-AnthG, or *N*²,*N*²-Me₂G, Fig. 2) was used to measure steady-state kinetic parameters (*k*_{cat} and *K*_m) for the incorporation of dCTP opposite G or these adducts by Dpo4 T239W, varying the concentration of dCTP (Table 2). As reported for the wild-type enzyme,^{35,36} the incorporation efficiencies were similar for *N*²-MeG and G and decreased as much as 80-fold for other *N*²-alkylG adducts and 160,000-fold for *N*²,*N*²-Me₂G, due to both the decrease in *k*_{cat} and increase in *K*_{m,dCTP}. As noted previously,³⁶ the trend was partially reversed with the bulky *N*²-AnthG.

Determination of *k*_{pol} and *K*_{d,dCTP} for dCTP incorporation by Dpo4 T239W

Dpo4 T239W showed burst kinetics for the incorporation of dCTP opposite G or *N*²-alkylG but no burst phase for incorporation opposite *N*²,*N*²-Me₂G, as observed with the wild-type enzyme^{35,36} (results not shown). Analysis of the changes in rates as a function of dCTP concentration (under conditions of excess Dpo4 T239W over oligonucleotide) yielded maximal rates of nucleotide incorporation (*k*_{pol}) and apparent dCTP binding affinity (*K*_{d,dCTP})^{37,38} (Fig. 3, Table 3, Supplementary Data Fig. S1). The incorporation efficiency (*k*_{pol}/*K*_{d,dCTP}) was decreased as a function of the bulk of the *N*²-alkylG adduct, with the exception of *N*²-AnthG (also noted in the steady-state measurements) (Table 2), similar to results observed with 24-mer/36-mer oligonucleotides and wild-type Dpo4.³⁶

Rapid fluorescence changes associated with dCTP binding to Dpo4 T239W-oligonucleotide complexes

Fluorescence changes were observed upon mixing dCTP opposite templates containing G and *N*²-alkylG adducts at varying dCTP concentrations (Fig. 4), consonant with previous results.³⁴ The use of 2',3'-dideoxy sugars precluded the formation of phosphodiester bonds, providing kinetic information prior to the step in which the phosphodiester bond is formed (Fig. 1, step 4). Addition of dCTP opposite G, *N*²-MeG, *N*²-IbG, and *N*²-BzG showed fast increases (Fig. 4a-d). Such changes have previously been attributed to conformational changes in the protein,³⁴ and rates were obtained by fitting the change of the fluorescence signal to a single exponential equation (Eq. 3) at each dCTP concentration. No detectable fluorescence changes were observed for the addition of dCTP opposite *N*²-NaphG, *N*²-AnthG, or *N*²,*N*²-Me₂G (Fig. 4e-g). (We checked the effect of the *N*²-NaphG and *N*²-AnthG oligomers on the steady-state (Trp) fluorescence of Dpo4 T239W, under the conditions used in Fig. 4, and found that none of the fluorescence (350 nm) was lost, similar to previous results with unmodified oligomers³⁴ (the *N*²-AnthG oligomer had major bands at 400-420 nm, in addition, but these did not interfere).

The dCTP ground dissociation constants (*K*_{d,dCTP,ground}) and dCTP-induced conformational change rate constants were estimated by fitting the observed rates (from the experiments with the 2', 3'-dideoxy oligonucleotides) against dCTP concentrations based on Eq. 4 in the following model³⁴ (Fig. 5 and Table 4)

(where "DNA" indicates the primer-template oligonucleotide complex). For the incorporation of dCTP opposite unmodified G, the ground-state dissociation constant (*K*_{d,dCTP,ground} = 250

$\pm 40 \mu\text{M}$), a forward conformational change rate ($k_3 = 46 \pm 5 \text{ s}^{-1}$), and a reverse conformational change rate ($k_{-3} = 9.6 \pm 2.1 \text{ s}^{-1}$) were all similar to previous results.³⁴ The addition of dCTP opposite N^2 -MeG, N^2 -IbG, or N^2 -BzG by Dpo4 T239W yielded similar kinetic parameters for dCTP binding and the induced conformational change (Table 4).

As shown previously with Dpo4 T239W using a normal primer (with 3'-OH),³⁴ the fluorescence signal (Trp) showed a rapid increase (within 0.5 s) and then slowly decreased for incorporation. The fast phase (increasing fluorescence) was previously concluded to correspond to a dCTP-induced conformational change (step 3 in Fig. 1) (following very rapid binding, step 2) and was observed with G, N^2 -MeG, N^2 -IbG, and N^2 -BzG but not any of the bulkier lesions (Fig. 6). The rates of dCTP-induced conformational change increased as a function of the dCTP concentration (Fig. 7), and fitting of the observed fast phase rates against dCTP concentrations according to Eq. 4 gave estimates of the fast phase rate constant (k_3) and its reverse rate constant (k_{-3}) by an independent method. The kinetic parameters obtained using this primer were similar to those obtained using the $^{13}\text{C}_{\text{dd}}$ -mer/18-mer oligonucleotide pair (Table 4), with one exception (k_3 for N^2 -BzG), indicating that the primer 3'-OH had little effect on dCTP binding and the conformational change of Dpo4 T239W.

The addition of dTTP opposite a $^{13}\text{C}_{\text{dd}}$ -mer/18-mer oligonucleotide pair containing G or an N^2 -alkylG adduct did not yield an increase in the fluorescence signal (Supplementary Data Fig. S2a), in contrast to the (correct) dCTP incorporation (Fig. 4a-d). The misincorporation of dTTP opposite G or any of the N^2 -alkylG adducts showed no kinetic burst phase of product formation (Supplementary Data Fig. S2b). Misincorporation of dTTP opposite G or any N^2 -alkylG adduct in a ^{13}C -mer/18-mer oligonucleotide pair (with free 3'-OH) also showed no increase in fluorescence (Supplementary Data Fig. S2c).

Discussion

N^2 -AlkylG adducts have been extensively studied with the replicative DNA polymerases bacteriophage pol T7⁻ and HIV-1 transcriptase and the translesion polymerases human DNA pols η , ι , κ , and Rev1, as well as Dpo4.^{35,36,39-43} Small lesions, even N^2 -MeG and N^2 -EtG, strongly block pol T7⁻ and HIV-1 reverse transcriptase,³⁹ in stark contrast to the relatively minor (or non-existent) inhibition of translesion polymerases. The Y-family DNA polymerases η , ι , κ , and Rev1 can all bypass the bulkier N^2 -alkylG adducts but the incorporation efficiency does decrease for the larger modifications.⁴⁰⁻⁴³ We have shown that wild-type Dpo4 is strongly blocked by N^2,N^2 -Me₂G³⁵ and bypasses N^2 -alkylG adducts, with the incorporation efficiency lowered by about ~120-fold.³⁶

Recently we developed the Dpo4 mutants T239W and N188W to monitor conformational changes associated with dNTP binding.³⁴ In this work Dpo4 T239W was used to analyze conformational changes associated with the addition of dCTP opposite N^2 -alkylG adducts (Fig. 2). A fluorescent signal that we attribute to a catalytically-relevant conformational change³⁴ was observed with G and adducts ranging in size up to N^2 -BzG. No fluorescence increases associated with conformational changes were observed for the N^2 -alkylG adducts bulkier than N^2 -BzG (N^2 -NaphG, N^2 -AnthG) or N^2,N^2 -Me₂G, consistent with abnormal crystal structures we have reported for N^2 -NaphG and N^2,N^2 -Me₂G.^{35,36} The incorporation of dCTP opposite G (and possibly the smaller N^2 -alkylG adducts) via standard W-C base pairing⁴⁴ induces a fast conformational change in Dpo4 T239W.³⁴ However, the crystal structures of Dpo4 with oligonucleotide duplexes containing N^2 -NaphG or N^2,N^2 -Me₂G showed disrupted, non-W-C base pairing.^{35,36} The fluorescent signal observed in the stopped-flow experiment is considered to be a result of the majority of Dpo4 molecules undergoing the same or at least a similar change in the orientation of Trp-239 upon binding of dCTP. The multiple binding orientations observed with N^2 -NaphG and N^2,N^2 -Me₂G^{35,36} are in agreement with the lack of a fluorescence signal

(Fig. 4e-g) reflecting the conformational change. As alternative abnormal binding modes are accommodated in the polymerase active site in solution, the amplification of the fluorescence signal will be diminished because these non-productive binding modes will not elicit the appropriate change in the conformation of Dpo4.

Earlier studies with the replicative DNA polymerases pol T7⁻ and *E. coli* pol I have been interpreted to indicate that the rate-limiting step is the conformational change for correct incorporations but changes to phosphodiester bond formation for misincorporations.^{13,37, 45-47} With a modified pol T7⁻, the conformational change acts as a molecular switch to discriminate matched dNTP from mismatched dNTPs.¹⁵ The directions of fluorescence changes (reflecting conformational changes) differed for correct and incorrect dNTP binding with coumarin-tagged pol T7⁻.³⁰ Thus, binding of the “wrong” dNTP is proposed to induce pol T7⁻ into a state that has higher energy barriers for proceeding to catalysis, thus favoring reversal of equilibrium events that would lead to phosphodiester bond formation. The results of computational calculations with pol β have been interpreted to suggest that correct incorporation is through a transition state with a closed conformation but that misincorporation occurs with one or several partially open conformations.⁴⁸ With *E. coli* pol I selection against mismatched dNTP has been proposed to occur in an “earlier” pathway (open state), and amismatched dNTP is proposed to accelerate DNA dissociation.²⁶

Our results suggest that the behavior of Dpo4 (T239W) may be different, probably because it is a translesion DNA polymerase (although pol β should not really be considered a replicative polymerase, in that it is primarily a gap filler). With Dpo4, small (*N*²-alkylG) DNA adducts that reduce catalytic efficiency by 60- to 80-fold (Tables 2, 3) still appear to produce the fluorescence changes associated with normal W-C pairing and replication (Figs. 4, 6) and the estimated rate constants are very similar to those measured with (unmodified) G (Figs. 5, 7 and Table 4). Thus, there seems to be little discrimination against the modification at G in the earlier stages of the mechanism (i.e., through step 3 in Fig. 1). This result appears to differ from the conclusion advanced for *E. coli* pol I, pol β , and pol T7⁻ (*vide supra*, although the point should be made that the focus with these polymerases is misincorporation). We presume the discrimination that lowers catalytic efficiency (Tables 2, 3) occurs in either step 4 (Fig. 1) (i.e. phosphodiester bond formation) or, alternatively, in the an altered partitioning into an inactive ternary complex, which we have proposed to account for the partial kinetic bursts observed (with wild type Dpo4).³⁶ In our earlier kinetic modeling studies³⁶ we could fit the varying kinetic bursts observed for dCTP incorporation opposite the *N*²-alkylG adducts by varying k_4 and holding k_3 , k_{-3} , and the ratio for partitioning into an inactive complex nearly constant with the *N*²-MeG, *N*²-EtG, *N*²-IbG, and *N*²-BzG adducts. At this point we are not able to further refine the relevant rate constants (other than k_3 and k_{-3} , Table 4) on the basis of experimental data. One caveat about our earlier modeling with wild-type Dpo4³⁶ is that the values of k_3 and k_{-3} we used there are much lower than the newer rate constants we have estimated with the T239W mutant,³⁴ and the values should probably be considered inappropriate.

With *N*²-alkylG adducts as large as *N*²-NaphG (or with *N*²,*N*²-Me₂G) no fluorescence changes were observed upon addition of dCTP (Fig. 4) and presumably the correct conformational change is blocked or at least very slow. This result is of interest in that the difference in catalytic efficiency of dCTP incorporation opposite *N*²-BzG and *N*²-NaphG was small (Tables 2, 3).

With some adducts the *anti/syn* conformation about template nucleosides or dNTPs can explain catalytic selectivity, e.g. with Dpo4 and 8-oxoG. In that case hydrogen bonding to the C8 oxygen (of 8-oxoG) drives the *anti/syn* equilibrium and accordingly the fidelity.⁴⁹⁻⁵¹ However, the conformational changes considered here are probably more complex. Crystal structures of

N^2 -NaphG³⁶ and N^2,N^2 -Me₂G³⁵ have shown both *anti* and *syn* conformations with no real correlation of either with catalytic activity.

Another point that should be made, relevant to the comparisons of Dpo4 (T239W) with *E. coli* pol I, pol β , and pol T7⁻, is that the focus of our work has been on the fluorescence/conformational changes observed with “normal” incorporation (of dCTP opposite G derivatives). For the addition of dTTP opposite any of the adducts there was no fluorescence change observed (Supplemental Data Fig. S2), and little can be said other than the correct conformational change appears not to occur. Whether an alternate (but silent) change occurs such as with pol T7^{-15,30} is unknown.

In summary, we have used fluorescent Dpo4 mutant T239W to show that a 2-order of magnitude loss of in catalytic efficiency of dCTP incorporation due to alkylation of the N2 atom of G by small moieties does not appear to involve changes in the rate or extent of dCTP-induced conformational changes, as judged by protein fluorescence measurements. Larger alkyl groups (N^2 -NaphG, N^2 -AnthG, and N^2,N^2 -Me₂G) blocked the fluorescence change, indicating that the conformation changes were strongly perturbed. Thus, modifications of different sizes at a single atom have multiple mechanisms of slowing DNA polymerization. In all of the cases examined, misincorporation (of a nucleoside triphosphate other than dCTP) is not preferred³⁶ and no signals were observed for fluorescence related to any conformational changes.

Materials and Methods

Materials

Unlabeled dNTPs were purchased from New England Biolabs (Beverly, MA), and [γ -³²P]ATP (specific activity 3×10^3 Ci mmol⁻¹) was from PerkinElmer Life Sciences (Boston, MA). T4 polynucleotide kinase and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Bio-spin columns were obtained from Bio-Rad (Hercules, CA). Other reagents were of the highest quality commercially available.

Dpo4 mutant T239W was expressed in *E. coli* and purified to electrophoretic homogeneity as described previously,^{34,52} except that the 80 °C heat step used for the wild-type enzyme was omitted because of the possibility of decreased mutant stability. Purified Dpo4 T239W was stored in small aliquots at -80 °C in 50 mM Tris-HCl buffer (pH 7.7 at 22 °C) containing 50 mM NaCl, 1 mM dithiothreitol, and 50% glycerol (v/v).

Oligonucleotides

Oligonucleotide complexes containing a 13-mer primer and an 18-mer template were used in this study (Table 1, Fig. 1). For analysis of fluorescence changes in the absence of phosphodiester bond formation, the 3'-end of the primer strand was rendered incapable of phosphoryl transfer by substituting a 2',3'-dideoxy analog (labeled “dd” in the text). For the analysis of fluorescence changes under conditions where phosphodiester bond formation can occur and the steady-state and pre-steady-state reactions in which oligonucleotide products were measured, the 13-mer primer contained the normal 3'-OH group.

The unmodified 13-mer and 18-mer and two 18-mer oligonucleotides containing N^2 -MeG or N^2 -IbG (Table 1) were synthesized and purified using HPLC by Midland Certified Reagent Co. (Midland, TX). The other four 18-mers, each containing an N^2 -alkylG adduct (N^2 -BzG, N^2 -NaphG, N^2 -AnthG, or a N^2,N^2 -Me₂G (Fig. 1) were prepared as previously described, purified by electrophoresis, and characterized by capillary gel electrophoresis and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry.^{35,36,39-43} The extinction

coefficients for the oligonucleotides, estimated by the Borer method,⁵³ were: 13-mer, $\epsilon_{260} = 112 \text{ mM}^{-1} \text{ cm}^{-1}$; and 18-mer, $\epsilon_{260} = 157 \text{ mM}^{-1} \text{ cm}^{-1}$.³⁵

The 5' end of the 13-mer primer was labeled with [γ -³²P]ATP using T4 polynucleotide kinase at 37 °C for 30 min. After removal of excess ATP using a Bio-Spin 6 column (Bio-Rad, Hercules, CA), the labeled primer and the template (molar ratio 1:1) were heated at 95 °C for 5 min and then slowly cooled to room temperature to form the 13-mer/18-mer primer/template duplexes, which were used for steady-state and pre-steady-state kinetic experiments.

Reaction Conditions for Dpo4 T239W Assays and Product Analysis Methods

Standard oligonucleotide polymerization reactions with Dpo4 T239W included 50 mM Tris-HCl buffer (pH 7.5 at 25 °C) containing 50 mM NaCl, 5 mM dithiothreitol, and 5% (v/v) glycerol at 37 °C.³⁴ Although bovine serum albumin is often used in DNA polymerase assays, it was not added here because it contains two Trp residues that could interfere with the fluorescence of Dpo4 mutant T239W.³⁴ Removal of albumin was previously found not to affect the rate of DNA polymerization. All reactions were initiated by mixing dNTP-MgCl₂ (final MgCl₂ concentration of 5 mM) solution to preincubated enzyme-DNA mixtures. After reaction, 5 μ l aliquots were quenched with EDTA-formamide solution (50 μ l of 20 mM EDTA in 95% formamide (v/v), with 0.5% bromphenol blue (w/v) and 0.05% xylene cyanol (w/v)). Products were resolved using 20% polyacrylamide (w/v) denaturing gel electrophoresis (containing 8 M urea) and visualized and quantitated by phosphorimaging analysis using a Bio-Rad Molecular Imager FX instrument and Quantity One software.

Steady-state Kinetic Assays

Steady-state single-base incorporation experiments were performed by adding a single dNTP at varying concentrations (twelve points) (plus MgCl₂) to the Dpo4 T239W-oligonucleotide complexes, incubated in the reaction buffer. The molar ratio of Dpo4 to oligonucleotide was < 10%, and primer conversion to product was kept < 20% by adjusting the Dpo4 T239W concentration and incorporation time.⁵⁴ Reactions were quenched and products were analyzed and quantitated; graphs of the incorporation rates versus dNTP concentration were fit to a hyperbolic equation to yield k_{cat} and K_{m} values using nonlinear regression in GraphPad Prism Version 3.0 (GraphPad, San Diego, CA). The corresponding errors were also obtained to indicate the goodness of fit.

Pre-steady-state Kinetic Analysis

Rapid quench experiments were performed in a model RQF-3 KinTek Quench Flow Apparatus (KinTek, Austin, TX) with a 50 mM Tris-HCl (pH 7.4) aqueous solution in the drive syringes. Reactions with excess Dpo4 T239W were initiated by mixing 200 nM Dpo4/10 nM oligonucleotide mixtures (12.5 μ l) with dCTP (varying concentrations)-5 mM MgCl₂ complex (10.9 μ l). After the reactions were quenched by the addition of 0.6 M EDTA from the central syringe line after varying times, the products were analyzed and quantitated. Reactions with excess Dpo4 T239W were fit to Eq. 1 (where P is product and t is time) to obtain a burst amplitude A and burst rate k_{p} :

$$P=A(1-e^{-k_{\text{p}}t}) \quad (\text{Eq. 1})$$

Plots of k_{p} versus dCTP concentration were fit to a hyperbolic equation to estimate k_{pol} and $K_{\text{d,dCTP,app}}$:

$$k_p = k_{pol} [dCTP] / ([dCTP] + K_{d,dCTP,app}) \quad (\text{Eq. 2})$$

where k_{pol} is the maximal rate of nucleotide incorporation and $K_{d,dCTP,app}$ is the apparent equilibrium dissociation constant for dCTP in the activated form of polymerase-oligonucleotide-dNTP complex.^{37,38} All nonlinear regression analysis used GraphPad Prism Version 3.0, with corresponding errors included to indicate the goodness of fit.

Stopped-flow fluorescence measurements

An OLIS RSM-1000 spectrofluorimeter was used in the measurement of transient fluorescent assays. For optimal signal/noise ratios for observing changes in Trp fluorescence, 3.16 mm slits (corresponding to a 20 nm bandwidth) were employed along with both 335 nm long-pass (CVI Laser Corp., Albuquerque, NM) and 355 nm bandpass filters (Newport, Irvine, CA) attached to the sample photomultiplier tube in series. Fluorescence signals were collected in the ratio mode (sample/reference photomultiplier). $MgCl_2$ (5 mM) was included in both syringes. In typical experiments for measuring the effect of dNTP binding on fluorescence of Dpo4 T239W, one syringe included Dpo4 T239W and the oligonucleotide in 50 mM Tris buffer (pH 7.5 at 25 °C) containing 5 mM dithiothreitol and 5% glycerol (v/v), and the second syringe contained various concentrations of the correct dNTP in the same Tris buffer. After rapid mixing, the final concentration of Dpo4 T239W:oligonucleotide complex was 1.0 μ M. In all cases, standard assays were performed including all components except the reagent producing a change (e.g., dNTP in the case of fluorescence change dependence on dNTPs). The fluorescence traces shown in the figures are averages of eight independent mixing events.

Data analysis of stopped-flow fluorescence measurements

The rate of conformational change at different dCTP concentration was analyzed by non-linear regression fitting the fluorescence increase signal against time using the equation:

$$y = A(1 - e^{-k_{obs} t}) \quad (\text{Eq. 3})$$

where y is the fluorescence signal, A is the amplitude of the signal, k_{obs} is the observed rate, and t is time. k_{obs} values were plotted against the dCTP concentration using a hyperbolic equation (Eq. 4) to estimate the ground state dissociation constant of dCTP ($K_{d,dCTP,ground}$), maximal forward conformational change rate constant (k_3), and reverse conformational change rate constant (k_{-3}) (Fig. 1):

$$k_{obs} = k_3 [dCTP] / (K_{d,dCTP,ground} + [dCTP]) + k_{-3} \quad (\text{Eq. 4})$$

All nonlinear regression analysis used the OLIS RSM-1000 software and GraphPad Prism Version 3.0, with corresponding errors indicating the goodness of fit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Anth	(9-anthracenyl)methyl
Bz	benzyl
Dbh	DinB homologue of <i>Sulfolobus acidocaldarius</i> (Dpo4 ortholog)
Dpo4	<i>Sulfolobus solfataricus</i> P2 DNA polymerase IV
dNTP	2'-deoxyribonucleoside triphosphate

Et	ethyl
G	guanine
Ib	isobutyl
Me	methyl
Naph	(1-naphthyl)methyl
PP _i	pyrophosphate
pol	(DNA) polymerase
W-C	Watson-Crick (base pair). For simplicity the <i>N</i> ² -guanyl adducts are usually collectively referred to as “alkyl” although technically some are aralkyl

Fig. 1. General DNA polymerization mechanism. E: polymerase, D_n : DNA (oligonucleotide) substrate; E^* : conformationally-modified polymerase; D_{n+1} : DNA (oligonucleotide) extended by one base (product); PP_i : pyrophosphate. Numbers of steps are associated with nomenclature of the rate constants.

Fig. 2.
Structures of N²-alkylG adducts used in this study.

Fig. 3. Estimation of k_{pol} and apparent $K_{d,dCTP}$ for the incorporation of dCTP opposite G and N^2 -alkylG adducts in 13C-mer/18-mer oligonucleotide pairs catalyzed by Dpo4 T239W. Dpo4 T239W (200 nM) was incubated with 10 nM 13C-mer/18-mer complexes in a rapid quench-flow instrument and then mixed with varying concentrations of dCTP (plus 5 mM Mg^{2+}) to initiate reactions. Plots of burst rates (k_{obs}) versus $[dCTP]$ were fit to a hyperbolic equation to yield k_{pol} and apparent $K_{d,dCTP}$ (values shown in figures).

Fig. 4.

Stopped-flow fluorescence changes for the addition of dCTP opposite 13C_{dd}-mer/18-mer oligonucleotide pair containing G or N²-alkylG adducts by Dpo4 T239W. The reactions included a complex of 1 μ M Dpo4 T239W–13C_{dd}-mer/18-mer oligonucleotide pair, 5 mM Mg²⁺, and varying concentrations of dCTP at 37 °C. dCTP concentrations are shown at the right of each part of the figure.

Fig. 5. Estimation of forward and reverse conformational change rate constants (k_3 and k_{-3}) and ground state $K_{d,dCTP}$ for the incorporation of dCTP opposite G or N^2 -alkylG adducts in 13C_{dd}-mer/18-mer oligonucleotide pairs by fitting the observed fluorescence change rates (Fig. 4) vs. dCTP concentrations (Eq. 4).

Fig. 6. Stopped-flow fluorescence changes (fast phase) for the addition of dCTP opposite G or N^2 -alkylG adducts in 13C-mer/18-mer oligonucleotide pairs by Dpo4 T239W. The conditions included 1 μ M Dpo4 T239W–13C-mer/18-mer oligonucleotide complex, 5 mM Mg^{2+} , and varying concentrations of dCTP at 37 °C. dCTP concentrations are shown at the right of each part of the figure.

Fig. 7.

Estimation of rate constants for conformational change and $K_{d,dCTP}$ following the addition of dCTP opposite G and N²-alkyl G adducts in 13C-mer/18-mer oligonucleotides. The rate constants, designated $k_{3'}$ and $k_{-3'}$ here (to distinguish from k_3 and k_{-3} estimated with dideoxy oligonucleotide, Figs. 4, 5), were estimated by fitting the observed fluorescence change rates against dCTP concentration.

Table 1

Oligodeoxynucleotides used in this study

13C _{dd} -mer	5'-GGGGGAAGGATTC _{dd} - 3'
13C-mer	5'-GGGGGAAGGATTC - 3'
18-mer	3'-CCCCCTTCCTAAGG*CACT- 5'

G*: G, *N*²-MeG, *N*²-IbG, *N*²-BzG, *N*²-NaphG, *N*²-AnthG, or *N*²,*N*²-Me₂G. Cdd: terminal 2',3'-dideoxycytidine.

Table 2Steady-state kinetic parameters for dCTP incorporation by Dpo4 T239W^a

Template base	k_{cat} s^{-1}	$K_{\text{m,dCTP}}$ μM	$k_{\text{cat}}/K_{\text{m,dCTP}}$ $\text{mM}^{-1} \text{s}^{-1}$	$k_{\text{cat}}/K_{\text{m,dCTP}}$ relative to G
G	0.057 ± 0.001	5.9 ± 0.5	9.7	1.0
<i>N</i> ² -MeG	0.078 ± 0.003	9.7 ± 1.8	8.0	0.82
<i>N</i> ² -IbG	0.011 ± 0.001	105 ± 30	0.10	0.010
<i>N</i> ² -BzG	0.0061 ± 0.0003	36 ± 6	0.17	0.018
<i>N</i> ² -NaphG	0.015 ± 0.001	124 ± 16	0.12	0.012
<i>N</i> ² -AnthG	0.0031 ± 0.001	5.1 ± 0.6	0.61	0.062
<i>N</i> ² , <i>N</i> ² -Me ₂ G	$(11 \pm 0.1) \times 10^{-6}$	190 ± 44	5.8×10^{-5}	5.9×10^{-6}

^aThe extent of conversion of primer to the product was kept < 20% by adjustment of the enzyme concentration and reaction time.

Table 3

Pre-steady-state kinetic analysis of dCTP incorporation by Dpo4 T239W

Template base	k_{pol} s^{-1}	$K_{\text{d,dCTP}}$ μM	$k_{\text{pol}}/K_{\text{d,dCTP}}$ $\text{mM}^{-1} \text{s}^{-1}$	$k_{\text{pol}}/K_{\text{d,dCTP}}$ relative to G
G	1.1 ± 0.1	35 ± 6	31	1.0
N^2 -MeG	2.8 ± 0.2	99 ± 23	28	0.90
N^2 -IbG	0.20 ± 0.01	89 ± 16	2.2	0.071
N^2 -BzG	0.48 ± 0.02	330 ± 40	1.5	0.15
N^2 -NaphG	0.26 ± 0.01	530 ± 60	0.49	0.016
N^2 -AnthG	0.70 ± 0.03	120 ± 16	5.8	0.19

Table 4
Pre-steady-state rate constants for conformational changes of Dpo4 T239W as detected by stopped-flow fluorescence

Template base	Basis of 2',3'-dideoxy oligonucleotide			Basis of normal (3'-OH) oligonucleotide		
	k_3 (s ⁻¹)	k_{33} (s ⁻¹)	k_3/k_{33}	K_d (μM)	$k_{3'}$ (s ⁻¹)	$k_{3'}$ (s ⁻¹)
G	46	9.6	4.8	250	29	9.2
N ² -MeG	12	5.2	2.3	43	11	5.2
N ² -IbG	27	13	2.1	170	15	11
N ² -BzG	33	6	5.5	250	8.9	7.6