Published in final edited form as:

Chembiochem. 2013 September 2; 14(13): 1634–1639. doi:10.1002/cbic.201300296.

Incorporation of Nucleoside Probes Opposite O⁶-Methylguanine by *Sulfolobus solfataricus* DNA Polymerase Dpo4: Importance of Hydrogen Bonding

Alessia Stornetta^a, Todor Angelov^a, F. Peter Guengerich^b, and Shana J. Sturla^{*,a}

^aDepartment of Health Sciences and Technology, Swiss Federal Institute of Technology Zurich, 8092 Zurich (Switzerland) ^bDepartment of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232-0146 (USA)

Abstract

O⁶-Methylguanine (O⁶-MeG) is a mutagenic DNA lesion, arising from the action of methylating agents on guanine (G) in DNA. Dpo4, an archaeal low-fidelity Y-family DNA polymerase involved in translesion DNA synthesis (TLS), is a model for studying how human Y-family polymerases bypass DNA adducts. Previous work showed that Dpo4-mediated dTTP incorporation is favoured opposite O⁶-MeG rather than opposite G. However, factors influencing the preference of Dpo4 to incorporate dTTP opposite O⁶-MeG are not fully defined. In this study, we investigated the influence of structural features of incoming dNTPs on their enzymatic incorporation opposite O⁶-MeG in a DNA template. To this end, we utilized a new fluorescence-based primer extension assay to evaluate the incorporation efficiency of a panel of synthetic dNTPs opposite G or O⁶-MeG by Dpo4. In single-dNTP primer extension studies, the synthetic dNTPs were preferentially incorporated opposite G relative to O⁶-MeG. Moreover, pyrimidine-based dNTPs were generally better incorporated than purine-based syn-conformation dNTPs. The results suggest that hydrophobicity of the incoming dNTP appears to have little influence on the process of nucleotide selection by Dpo4, with hydrogen bonding capacity being a major influence. Additionally, modifications at the C2-position of dCTP increase the selectivity for incorporation opposite O⁶-MeG without a significant loss of efficiency

Keywords

DNA damage; DNA polymerase; fluorescence; nucleotide analogs; translesion DNA synthesis

Introduction

Alkylation-induced DNA adducts are caused by chemicals from sources such as environmental pollutants, components of tobacco smoke, by-products of food processing, or anticancer drugs.^[1] Genetic mutations may arise from chemically modified DNA when an incorrect deoxyribonucleotide triphosphate is incorporated by DNA polymerases opposite an adduct.^[2] Among DNA alkylation adducts, O⁶-G-adducts, including O⁶-methylguanine (O⁶-MeG), are of toxicological relevance and are highly mutagenic.^[3] Despite the fact that O⁶-MeG is repaired by O⁶-alkylguanine DNA alkyltransferase,^[4] it can persist in DNA and, during DNA synthesis, polymerases tend to incorporate cytosine (C) or thymine (T) opposite O⁶-MeG with similar efficiency.^[2, 5] Thus, G:C to A:T transition mutations may arise.^[5d, 6]

Translesion DNA synthesis can maintain genomic integrity in the face of DNA lesions. During DNA synthesis, replicative polymerases, which are unable to replicate damaged templates, are blocked at the site of the lesion. To bypass this block, specialized TLS polymerases, capable of DNA synthesis over damaged DNA, are recruited to complement replicative polymerases and insert one or more dNTPs opposite the lesion. While allowing the progression of DNA synthesis, this process is potentially error-prone due to the low-fidelity of TLS polymerases.^[7] Many of these belong to the Y-family of polymerases.^[8] For *O*⁶-MeG, significant insight regarding Y-family polymerase-mediated DNA damage bypass has been gained by a combination of results with crystal structures of polymerases bound to modified DNA and kinetic characterization of the efficiency of nucleotide incorporation.^[9] In the active site of the enzyme, the presence of the methyl group impacts hydrogen bonding capacity, distribution of electron density, and base pair geometry, resulting in a wobble *O*⁶-MeG:C or pseudo-Watson-Crick *O*⁶-MeG:T base pair (Scheme 1).^[9]

The archaeal DNA polymerase Dpo4 is used widely as a model for studying how human Y-family polymerases bypass DNA adducts.^[9–10] Dpo4 is a low-fidelity thermostable enzyme. At 37 °C, it exhibits a misinsertion rate (for the four natural bases (A, C, T, and G)) in the range of 8×10^{-3} to 3×10^{-4} .^[11] Studies on the process of dNTP incorporation by Dpo4 for unmodified vs. *O*⁶-MeG-containing templates indicate that Dpo4 bypasses *O*⁶-MeG by inserting C in most cases (~70%), followed by T or A (~20% or less).^[9] Dpo4 also is able to accommodate unusual DNA structures in its active site.^[12]

Among chemical and biochemical factors controlling polymerase-mediated dNTP incorporation opposite DNA adducts are the identity of the adduct,^[2, 13] base-base interactions in the polymerase active site,^[14] base-protein interactions, and conformational changes of the protein after dNTP binding,^[15] and sequence context.^[16] A goal of this study was to examine the influence of individual incoming dNTP structural features on the efficiency of Dpo4-mediated nucleotide incorporation opposite *O*⁶-MeG. A motivation for obtaining this information is to understand chemical-level aspects of the TLS process, as well as to provide insight needed to engineer molecular strategies for detecting DNA adducts. We therefore tested the incorporation of a panel of synthetic dNTPs (Scheme 2) chosen on the basis of their structural similarities with dCTP and dTTP, the natural dNTPs best incorporated opposite *O*⁶-MeG. The contribution of hydrogen bonding potential, size, and hydrophobicity of the incoming dNTP (when inserted opposite G or *O*⁶-MeG) was also investigated.

To perform this study, we developed a fast, simple, and robust fluorescence-based assay for evaluating dNTP incorporation by a variety of DNA polymerase enzymes. The results indicate that modifications at defined positions of the incoming dNTP increase the selectivity for incorporation opposite *O*⁶-MeG.

Results

In order to assess the relative incorporation efficiency of modified dNTPs opposite G or *O*⁶-MeG, a panel of synthetic dNTPs was tested as substrates in single nucleotide primer extension reactions catalysed by Dpo4. A 15-mer primer (5'-CTCTAATCAGGGAGC-3') was annealed to a 35-mer template (5'-TTTCTTCTTCTTXGCTCCCTGATTAGAGTCGTAG-3'), in which X=G or *O*⁶-MeG, primer extension reactions were performed, the products separated using gels, and bands diagnostic of product formation were visualized with a fluorescent stain. The panel included dNTPs having the same or similar hydrogen bonding capacity as dCTP and dTTP, the two natural dNTPs best incorporated opposite *O*⁶-MeG. The panel included also dNTPs that

exhibited increased stability when paired with *O*⁶-MeG vs G in the context of duplex DNA (Scheme 2).

Single dNTP Primer Extension with Dpo4

dCTP and its derivatives 5-Me-dCTP, 2-thio-dCTP, *N*⁴-Me-dCTP, and dPTP (Scheme 2) were tested for incorporation by Dpo4 opposite G vs. *O*⁶-MeG. dCTP and 5-Me-dCTP were efficiently incorporated opposite a templating G, but significantly less so opposite a templating *O*⁶-MeG. 2-Thio-dCTP was efficiently inserted opposite G and also incorporated opposite *O*⁶-MeG, to a similar extent as dCTP. Of the dNTPs assayed, dCTP, 5-Me-dCTP, and 2-thio-dCTP displayed the highest incorporation efficiency opposite *O*⁶-MeG. *N*⁴-Me-dCTP, on the other hand, was incorporated to a significantly lower extent than either dCTP or 5-Me-dCTP opposite G, and its incorporation opposite *O*⁶-MeG was barely detectable. Finally, dPTP was incorporated opposite G slightly less efficiently than dCTP and was not incorporated opposite *O*⁶-MeG (Figure 1).

Dpo4-mediated incorporation opposite G and *O*⁶-MeG was also tested with dTTP, dUTP, and 4-thio-dTTP (Scheme 2). Significantly, dTTP incorporation opposite *O*⁶-MeG was more efficient than opposite G. dUTP incorporation opposite G was similar to that of dTTP, but its incorporation opposite *O*⁶-MeG was less efficient than that of dTTP. The dTTP-analog 4-thio-dTTP was not incorporated opposite either template by Dpo4 (Figure 1).

Dpo4-mediated incorporation opposite G vs. *O*⁶-MeG was also tested with 8-oxo-dATP and 8-Br-dATP (Scheme 2). These two dATP analogs were not inserted opposite *O*⁶-MeG and were only slowly incorporated opposite G (Figure 1). 8-Oxo-dATP and 8-Br-dATP, together with 4-thio-dTTP, were overall the least efficiently incorporated within the panel.

It is well established that Mn²⁺, with its relaxed coordination requirements, enhances both misincorporation and translesion synthesis by Dpo4.^[17] Therefore, in an effort to increase the incorporation of the poorly incorporated dTTP, dUTP, 4-thio-dTTP, 8-Br-dATP, and 8-oxo-dATP and to determine relative rates of their incorporation, we substituted Mg²⁺ with Mn²⁺. All of the previously unsuccessful reactions proceeded and dNTP analogs were inserted opposite both G and *O*⁶-MeG, but always more efficiently for G-containing templates (supplemental Figure S1). However, the selectivity profile was too different between the two ions to be informative regarding the TLS mechanism and we did not explore the kinetics of dNTP incorporation in the presence of Mn²⁺ any further. Additionally, increasing the reaction temperature to 60 °C did not promote previously unsuccessful insertions (supplemental Figure S2).

Steady-State Kinetics of dCTP, 5-Me-dCTP, 2-thio-dCTP and dPTP Incorporation by Dpo4

The kinetic parameters $k_{\text{cat,dNTP}}$ and $K_{\text{m,dNTP}}$ and the relative efficiency of dNTP incorporation were determined for dCTP, 5-Me-dCTP, 2-thio-dCTP, and dPTP, the four nucleotides for which measurable incorporation was observed opposite G or *O*⁶-MeG (Table 1). dNTP incorporation efficiencies varied for dCTP and its synthetic derivatives 5-Me-dCTP, 2-thio-dCTP and dPTP. Thus, dCTP was incorporated 10³-fold less efficiently opposite *O*⁶-MeG than opposite G, but still had the highest incorporation efficiency opposite *O*⁶-MeG among the dNTPs tested. While dCTP and 5-Me-dCTP were incorporated with similar efficiency opposite G, 5-Me-dCTP was incorporated 1.7-fold less effectively than dCTP opposite *O*⁶-MeG. For 2-thio-dCTP, incorporation was 50- and 10³-fold less efficient than dCTP opposite G or *O*⁶-MeG, respectively. Among the four dNTPs, which were analysed, dPTP was the only dNTP not inserted opposite *O*⁶-MeG; it was inserted opposite G 80-fold less efficiently than was dCTP (Table 1).

Discussion

*O*⁶-MeG adducts in DNA are well established as mutagenic and result in G-to-A transition mutations.^[5a, 5d, 6] The chemical and biochemical bases of how mutations arise from *O*⁶-MeG relate to the fidelity and efficiency of DNA polymerases that encounter this DNA adduct. DNA polymerases from different families process *O*⁶-MeG adducts in a unique manner. The A-family replicative polymerases *Bf*,^[5a] *Kf*, *Kf*(exo-), *Taq*, and *Tth*^[2] were shown to be two to five orders of magnitude less efficient at incorporating dCTP opposite *O*⁶-MeG than opposite G. Except for *Kf*(exo-), all of them displayed also a strong preference to misincorporate T, and not C, opposite *O*⁶-MeG.^[2, 5a] Two B-family polymerases, *Pol* δ and bacteriophage T4 DNA polymerase, were also shown to suffer a reduced C incorporation efficiency opposite *O*⁶-MeG (10- and 1000-fold, respectively).^[13, 18] In contrast to polymerases of the A-family, these two B-family polymerases had equal C and T incorporation efficiencies opposite *O*⁶-MeG.^[13, 18]

Two different approaches have been used to elucidate the factors contributing to *O*⁶-MeG bypass by replicative polymerases. The first one, based on the use of *O*⁶-MeG analogs with altered Watson-Crick hydrogen bonding sites, has been used to identify hydrogen bonding interactions between dTTP or dCTP and *O*⁶-MeG and their importance for DNA replication by A-family polymerases. The authors of that study identified hydrogen bonding interactions with the N1 position and the exocyclic amino group of *O*⁶-MeG to be critical for dTTP and dCTP incorporation, respectively.^[2] A different approach, based on the use of non-natural dNTPs, was used in a study investigating the relative roles of hydrogen-bonding interactions, shape/size, and nucleobase desolvation during DNA replication. The authors concluded that in the case of T4 DNA polymerase, hydrophobicity and shape complementarity are the major factors that dictate dNTP incorporation opposite *O*⁶-MeG.^[13]

Y-family polymerases, the main function of which is DNA lesion bypass, vary both in their T-opposite-*O*⁶-MeG misinsertion frequencies and the extent with which they are inhibited by *O*⁶-MeG.^[9, 18–19] The thermostable Y-family polymerase Dpo4 is one of the rare examples among TLS and replicative polymerases that displays a preference for correct dCTP incorporation opposite *O*⁶-MeG.^[9] In the active site of Dpo4, *O*⁶-MeG and C form a wobble base pair, whereas *O*⁶-MeG and T form a Watson-Crick-like base pair (Scheme 1).^[9] An aim of the current study was to further develop this model by evaluating the role that more varied structural features of incoming dNTPs play in their selection by Dpo4 for incorporation opposite a templating *O*⁶-MeG.

Steady-state primer extension kinetic assays are generally performed with primer strands radioactively labelled at their 5'-end with [γ -³²P]-ATP,^[20] and products are separated by denaturing polyacrylamide gel electrophoresis on large gel plates (30 × 40 cm × 0.4 mm), with the resulting bands visualized with a phosphorimager.^[20] We applied a new analysis approach amenable to more rapid testing of dNTPs as TLS substrates, which avoids the use of radioactivity. To separate DNA bands, we used small gels (8 × 8 cm × 1 mm), which also shortened the gel electrophoresis time. The small gels are not ideal for primers longer than 20 bases, but for primers with fewer than 20 bases 20% acrylamide (w/v)/7 M urea gels offer good resolution between primer and extension products. Product bands were detected by fluorescence labelling with SYBR Gold,^[21] thus avoiding radioactive labelling and autoradiography.

Using this technique, we obtained steady-state kinetic parameters for Dpo4-mediated dCTP incorporation opposite G vs. *O*⁶-MeG consistent with previously published data,^[9] confirming the relative efficiency of dCTP incorporation opposite *O*⁶-MeG vs. G to be 1:1000 (Table 1). Additionally, our data confirmed that Dpo4 displays a preference for T-

misincorporation opposite O^6 -MeG and incorporates T more efficiently opposite O^6 -MeG than opposite G (Figure 1).^[9]

We applied the fluorescence-based approach to investigate structure-activity relationships for dNTP incorporation opposite O^6 -MeG, first considering the potential influence of changes on the non-hydrogen bonding face of the incoming dNTP. We investigated Dpo4-mediated incorporation efficiencies of dUTP, dTTP, dCTP and 5-Me-dCTP (Scheme 2). Relative to their unmethylated analogs (dUTP and dCTP), dTTP and 5-Me-dCTP were equally well incorporated opposite the G-containing template. However, opposite O^6 -MeG, dTTP appeared to be slightly more efficiently incorporated than dUTP (Figure 1), whereas steady-state kinetic data indicated that 5-Me-dCTP was 1.7-fold less efficiently incorporated than dCTP opposite O^6 -MeG (Table 1). The influence of C5-methylation of thymine and cytosine on their incorporation opposite O^6 -MeG by Kf(exo-) was previously investigated, and it was found that the additional hydrophobic surface of the methyl group plays a minor role in dNTP selection and incorporation.^[2] Despite the differences between Dpo4 and Kf(exo-), our results show that, similarly to the situation with Kf(exo-), methyl substitution of the incoming nucleotide has little influence on dNTP selection and incorporation.

Only the O2 and N3 atoms of the thymine base have been shown to be involved in direct interactions with the opposite base upon base-pairing with G and O^6 -MeG.^[9] The carbonyl group at C4 position, however, may influence the efficiency of dTTP incorporation opposite G and O^6 -MeG through indirect mechanisms not involving hydrogen bonding with the base-pairing partner of T. To investigate this possibility we evaluated the incorporation of 4-thio-dTTP opposite G and O^6 -MeG. Its hydrogen bonding properties are unchanged relative to dTTP. We found that 4-thio-dTTP is incorporated opposite only G, with low efficiency (Figure 1). The increased size and lower electronegativity of sulfur vs. oxygen may contribute to steric clash or impaired hydrogen bonding between the thiocarbonyl group and the polymerase active site. These results suggest that increased size and decreased electronegativity of 4-thio-dTTP vs. dTTP is an important contributing factor for its inefficient insertion opposite G and O^6 -MeG.

To test potential influences of hydrogen bonding on dNTP selection by Dpo4 for incorporation opposite G vs. O^6 -MeG, we also studied the incorporation of dPTP and 2-thio-dCTP. dPTP has two tautomeric forms with the potential to hydrogen bond similar to either C or T (Scheme 2).^[22] We found that dPTP was incorporated opposite G with 80-fold lower efficiency than dCTP, but not incorporated opposite O^6 -MeG (Figure 1 and Table 1). In a previous study in which the efficiency of dPTP incorporation opposite G vs. O^6 -MeG with the bacteriophage T4 DNA polymerase was investigated, similar results were obtained. Thus, dPTP was incorporated opposite G, but its efficiency of incorporation opposite O^6 -MeG was 1.5- and 7-fold lower than that of dCTP and dTTP, respectively.^[13] These results are consistent with dPTP being present in the Dpo4 active site predominantly in its dCTP-like tautomeric form, i.e. properly hydrogen bonding, but undergoing unfavourable steric interactions with the enzyme. As such dPTP displays the same selectivity as dCTP, but its incorporation efficiency is always diminished. In the case of 2-thio-dCTP, on the other hand, modified dNTP incorporation opposite the adduct was competitive with dCTP incorporation. 2-Thio-dCTP incorporation opposite G could be expected to be impaired due to lack of hydrogen bonding complementarity and the increased size associated with the thiocarbonyl group. Indeed, compared to dCTP, 2-thio-dCTP was 50-fold less efficiently incorporated opposite G. However, the efficiency of 2-thio-dCTP incorporation opposite O^6 -MeG was identical to that of dCTP (Table 1). This phenomenon may be explained on the basis of dCTP and 2-thio-dCTP interacting similarly with O^6 -MeG in the Dpo4 active site. The O2 oxygen atom of dCTP is not involved in hydrogen-bonding interactions when paired with

*O*⁶-MeG (Scheme 1).^[9] The overall impact of the 2-thio modification was, therefore, loss in selectivity for incorporation opposite G (Figure 2).

Finally, 8-Br-dATP and 8-oxo-dATP, two purine dNTPs displaying a *syn*-conformational preference, were included in our panel because they formed more stable base pairs with *O*⁶-MeG than with G in duplex DNA¹. 8-Br-dATP is more hydrophobic than 8-oxo-dATP, and likely unable to properly hydrogen bond with either G or *O*⁶-MeG, whereas 8-oxo-dATP is more polar, and may hydrogen bond through its 8-oxo and 7-imino groups with *O*⁶-MeG. Thus, we viewed 8-Br-dATP and 8-oxo-dATP as a pair of non-pyrimidine base-pairing partners sterically acceptable for G and *O*⁶-MeG, which due to their dissimilar hydrophobicity and hydrogen bonding potential could provide complementary information on the relative importance of hydrophobicity vs. hydrogen bonding in dNTP incorporation by Dpo4. Both 8-Br-dATP and 8-oxo-dATP were, however, poorly incorporated opposite G and not incorporated opposite *O*⁶-MeG at all (Figure 1).

Conclusion

The modified dNTPs tested in this study were preferentially incorporated opposite G relative to *O*⁶-MeG, but we identified factors with the potential to change this selection gap. Thus, while changing the 4-substituent of dCTP from a hydrogen bond donor to an acceptor strongly reduces incorporation efficiency opposite both G and *O*⁶-MeG, reducing the hydrogen bond acceptor capacity at the O2 atom of dCTP, on the other hand, only reduces incorporation efficiency opposite G but not *O*⁶-MeG. For example, 2-thio-dCTP displays, relative to dCTP, an overall improved selectivity for *O*⁶-MeG, without significant loss of incorporation efficiency (Figure 2). Overall, these results provide insight regarding chemical factors that either impact or have little influence on the error propensity of *O*⁶-MeG bypass and also suggest that reducing hydrogen-bond acceptor capacity at the O2 atom of dCTP may be a viable strategy for promoting dNTP incorporation opposite *O*⁶-MeG or related adducts.

Experimental Section

Materials

Recombinant Dpo4 was heterologously expressed in *Escherichia coli* and purified as previously described.^[10b] The 15-mer primer and the 35-mer template sequences were 5'-CTCTAATCAGGGAGC-3' and 5'-TTTCTTCTTCTTTXGCTCCCTGATTAGAGTCGTAG-3', in which X=G or *O*⁶-MeG. Unmodified oligonucleotides were obtained, HPLC-purified, from VBC-Biotechnology (Vienna, Austria). The *O*⁶-MeG-containing template was synthesized on a MerMade-4 DNA oligonucleotide synthesizer (BioAutomation, Irving, TX) with standard phosphoramidate chemistry and deprotection procedures, and it was purified by reversed-phase HPLC on an Agilent XDB-Eclipse C18 column (4.5 mm × 150 mm, 4 μm particles) with a linear gradient of 5–15% acetonitrile in aqueous triethylammonium acetate (50 mM, pH 7). The identity of the template was confirmed by ESI-MS ([M-H][−] calculated: 10688.9; found: 10688.4) on a Velos LTQ instrument from Thermo Scientific (Waltham, MA). dTTP and dCTP were purchased from New England Biolabs (Ipswich, MA). dUTP, 8-Br-dATP, 5-Me-dCTP, and dPTP were purchased from Jena Bioscience (Jena, Germany). 2-Thio-dCTP, *N*⁴-Me-dCTP, 4-thio-dTTP, and 8-oxo-dATP were purchased from Trilink Biotechnologies (San Diego, CA). SYBR Gold was purchased from Life Technologies (Carlsbad, CA).

¹Angelov, T., and Sturla, S.J., unpublished results.

Single Nucleotide Primer Extension

All reactions were performed at 37 or 60 °C in solutions comprised of Tris-HCl buffer (50 mM, pH 7.4), NaCl (50 mM), DTT (5 mM), MgCl₂ (5 mM) or MnCl₂ (5 mM), bovine serum albumin (100 µg/ml), and 5% glycerol (v/v). Dpo4 (44 nM) was pre-incubated with pre-annealed primer-template dsDNA (2.5 µM), and the reaction was initiated by the addition of dNTP solution (90 µl total volume, and a final dNTP concentration ranging from 400 to 800 µM). A single aliquot (14 µl) was removed from each reaction mixture at 45 min and quenched with a solution of urea (20 µl total volume, 7 M) containing EDTA (20 mM, pH 8.0). The progress of the enzymatic reaction was monitored by gel electrophoresis on a 20% (w/v) acrylamide/ urea (7 M) gel with an XCell SureLock™ mini-cell electrophoresis system from Invitrogen (Paisley, UK). DNA bands were stained with SYBR Gold^[21] by incubating the gel in a 1× dye solution for 20 min upon gentle shaking at room temperature. The fluorescent DNA bands were visualized with a Molecular Imager Gel Doc™ XR+ Imaging System from Bio-Rad (Hercules, CA).

Steady-State Kinetic Analysis of dCTP, 5-Me-dCTP, 2-thio-dCTP and dPTP Incorporation

All reactions were performed at 37 °C in a solution comprised of Tris-HCl buffer (50 mM, pH 7.4), NaCl (50 mM), DTT (5 mM), MgCl₂ (5 mM), bovine serum albumin (100 µg/ml), and 5% (v/v) glycerol. A range of different dNTP concentrations was tested. Dpo4 (44 nM) was pre-incubated with pre-annealed primer-template dsDNA (2.5 µM), and the reaction was initiated by the addition of dNTP solution (90 µl total volume). The time range for which product formation was linear with respect to time (less than 20% of product formation) was determined, and a single aliquot (14 µl) was removed from the reaction mixture at a time point within the linear phase of the reaction and quenched with a solution urea (20 µl, 7 M) containing EDTA (20 mM, pH 8.0). Bands corresponding to DNA product and substrate were separated by gel electrophoresis and visualized as described above; band intensities were quantified with Image Lab™ Software (version 3.0). Concentrations were determined relative to the initial primer concentration ($[\text{primer}]_0 = [\text{primer}]_{\text{final}} + [\text{product}]_{\text{final}}$). Reaction rates were plotted as a function of dNTP concentration, and data were fit to hyperbolae with Sigma Plot (version 12.2) to estimate $k_{\text{cat,dNTP}}$ and $K_{\text{m,dNTP}}$. All values after “±” in Table 1 represent standard deviation from three independent experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the European Research Council (260341) and United States Public Health Service Grant R01 ES010375.

Abbreviations

2-Thio-dCTP	2-thio-deoxycytidine triphosphate
4-thio-dTTP	4-thio-deoxythymidine triphosphate
5-Me-dCTP	5-methyl-deoxycytidine triphosphate
8-Br-dATP	8-bromo-deoxyadenosine triphosphate
8-oxo-dATP	8-oxo-deoxyadenosine triphosphate
A	adenine

BF	Bacillus fragment
C	cytosine
Dpo4	thermostable DNA polymerase IV of <i>Sulfolobus solfataricus</i>
DTT	dithiothreitol
dPTP	6 <i>H</i> ,8 <i>H</i> -3,4-dihydro-pyrimido[4,5- <i>c</i>][1,2]oxazin-7-one-8-β-D-2'-deoxyribofuranosid-5'-triphosphate
G	guanine
Kf	Klenow fragment of <i>Escherichia coli</i> DNA polymerase I
N4-Me-dCTP	N4-methyl-deoxycytidine triphosphate
MeG	methylguanine
T	thymine
Taq	thermostable DNA polymerase of <i>Thermus aquaticus</i>
TLS	translesion synthesis
Tth	thermostable DNA polymerase of <i>Thermus thermophilus</i>
U	uracil

References

1. a) Drabløs F, Feyzi E, Aas PA, Vaagbø CB, Kavli B, Bratlie MS, Peña-Díaz J, Otterlei M, Slupphaug G, Krokan HE. DNA Repair. 2004; 3:1389–1407. [PubMed: 15380096] b) Dipple A. Carcinogenesis. 1995; 16:437–441. [PubMed: 7697795]
2. Spratt TE, Levy DE. Nucleic Acids Res. 1997; 25:3354–3361. [PubMed: 9241252]
3. Margison GP, Santibáñez Koref MF, Povey AC. Mutagenesis. 2002; 17:483–487. [PubMed: 12435845]
4. a) Demple B, Jacobsson A, Olsson M, Robins P, Lindahl T. J. Biol. Chem. 1982; 257:13776–13780. [PubMed: 6754717] b) Lindahl T, Sedgwick B, Sekiguchi M, Nakabeppu Y. Annu. Rev. Biochem. 1988; 57:133–157. [PubMed: 3052269]
5. a) Warren JJ, Forsberg LJ, Beese LS. Proc. Natl. Acad. Sci. U.S.A. 2006; 103:19701–19706. [PubMed: 17179038] b) Loechler EL, Green CL, Essigmann JM. Proc. Natl. Acad. Sci. U.S.A. 1984; 81:6271–6275. [PubMed: 6093094] c) Woodside AM, Guengerich FP. Biochemistry. 2001; 41:1027–1038. [PubMed: 11790127] d) Snow ET, Foote RS, Mitra S. J. Biol. Chem. 1984; 259:8095–8100. [PubMed: 6376499]
6. Pauly GT, Moschel RC. Chem. Res. Toxicol. 2001; 14:894–900. [PubMed: 11453737]
7. Lehmann AR, Niimi A, Ogi T, Brown S, Sabbioneda S, Wing JF, Kannouche PL, Green CM. DNA Repair. 2007; 6:891–899. [PubMed: 17363342]
8. a) Ohmori H, Friedberg EC, Fuchs RPP, Goodman MF, Hanaoka F, Hinkle D, Kunkel TA, Lawrence CW, Livneh Z, Nohmi T, Prakash L, Prakash S, Todo T, Walker GC, Wang Z, Woodgate R. Mol. Cell. 2001; 8:7–8. [PubMed: 11515498] b) Plosky BS, Woodgate R. Curr. Opin. Genetics Dev. 2004; 14:113–119.
9. Eoff R, Irimia A, Egli M, Guengerich FP. J. Biol. Chem. 2007; 282:1456–1467. [PubMed: 17105728]
10. a) Ling H, Boudsocq F, Woodgate R, Yang W. Cell. 2001; 107:91–102. [PubMed: 11595188] b) Zang H, Goodenough AK, Choi JY, Irimia A, Loukachevitch LV, Kozekov ID, Angel KC, Rizzo CJ, Egli M, Guengerich FP. J. Biol. Chem. 2005; 280:29750–29764. [PubMed: 15965231] c) Zang H, Irimia A, Choi J-Y, Angel KC, Loukachevitch LV, Egli M, Guengerich FP. J. Biol. Chem. 2006; 281:2358–2372. [PubMed: 16306039]

11. Boudsocq F, Iwai S, Hanaoka F, Woodgate R. *Nucleic Acids Res.* 2001; 29:4607–4616. [PubMed: 11713310]
12. Rechakoblit O, Malinina L, Cheng Y, Kuryavyy V, Broyde S, Geacintov NE, Patel DJ. *PLoS Biol.* 2006; 4:e11. [PubMed: 16379496]
13. Chavarria D, Ramos-Serrano A, Hirao I, Berdis AJ. *J. Mol. Biol.* 2011; 412:325–339. [PubMed: 21819995]
14. Kool ET. *Annu. Rev. Biochem.* 2002; 71:191–219. [PubMed: 12045095]
15. Johnson KA. *Annu. Rev. Biochem.* 1993; 62:685–713. [PubMed: 7688945]
16. Singer B, Chavez F, Goodman MF, Essigmann JM, Dosanjh MK. *Proc. Natl. Acad. Sci. U.S.A.* 1989; 86:8271–8274. [PubMed: 2682644]
17. a) Vaisman A, Ling H, Woodgate R, Yang W. *EMBO J.* 2005; 24:2957–2967. [PubMed: 16107880] b) Junop MS, Haniford DB. *EMBO J.* 1996; 15:2547–2555. [PubMed: 8665862] c) Zamft BM, Marblestone AH, Kording K, Schmidt D, Martin-Alarcon D, Tyo K, Boyden ES, Church G. *PLoS One.* 2012; 7:e43876. [PubMed: 22928047]
18. Choi J-Y, Chowdhury G, Zang H, Angel KC, Vu CC, Peterson LA, Guengerich FP. *J. Biol. Chem.* 2006; 281:38244–38256. [PubMed: 17050527]
19. Choi J-Y, Guengerich FP. *J. Biol. Chem.* 2008; 283:23645–23655. [PubMed: 18591245]
20. Boosalis MS, Petruska J, Goodman MF. *J. Biol. Chem.* 1987; 262:14689–14696. [PubMed: 3667598]
21. Tuma RS, Beaudet MP, Jin X, Jones LJ, Cheung C-Y, Yue S, Singer VL. *Anal. Biochem.* 1999; 268:278–288. [PubMed: 10075818]
22. Lin PKT, Brown DM. *Nucleic Acids Res.* 1989; 17:10373–10383. [PubMed: 2602155]

Figure 1.

Single primer extension with unmodified and *O*⁶-MeG-containing template catalysed by Dpo4 at 37 °C with magnesium in the reaction buffer. Oligonucleotide product length is shown on the right of the gel image. The reaction time was 45 min. Final [dNTP]_G: 400 μM, final [dNTP]_{O⁶-MeG}: 600 or 800 μM.

Figure 2.

Selectivity factor of steady-state kinetic parameters for dCTP, 5-Me-dCTP, 2-thio-dCTP, or dTTP for incorporation opposite *O*⁶-MeG versus G. The selectivity factor is calculated as $(k_{\text{cat}}/K_{\text{m}})_{O^6\text{-MeG}}/(k_{\text{cat}}/K_{\text{m}})_{\text{G}}$. A selectivity factor > 1 means that the dNTP is preferentially incorporated opposite *O*⁶-MeG.

^aCalculated as the ratio between $(k_{\text{cat}}/K_{\text{m}})_{O^6\text{-MeG}}$ and $(k_{\text{cat}}/K_{\text{m}})_{\text{G}}$ reported by Eoff et al.^[9]

Scheme 1.

Wobble $O^6\text{-MeG:C}$ (left) and pseudo-Watson-Crick $O^6\text{-MeG:T}$ base pairs (right).

R=deoxyribose in DNA strand.^[9]

Scheme 2.

Natural and non-natural dNTPs evaluated in this study. dR= deoxyribose triphosphate.

Table 1

Steady-state kinetics for single nucleotide primer extension catalysed by Dpo4.

dNTP	Template	k_{cat} [s ⁻¹]	$K_{m,dNTP}$ [μM]	Ratio of k_{cat}/K_m for dCTP:G vs. entry	$(k_{cat}/K_m)_{G,dNTP}$ $(k_{cat}/K_m)_{O^6-MeG,dNTP}$
dCTP	G	0.28 ± 0.04	10.1 ± 1.2	1	1000
	O ⁶ -MeG	0.02 ± 0.001	773 ± 131	1000 ^[a]	
5-Me-dCTP	G	0.33 ± 0.08	13.1 ± 3.9	1	1600
	O ⁶ -MeG	0.02 ± 0.003	1220 ± 160	1800	
2-thio-dCTP	G	0.04 ± 0.01	66.8 ± 18.7	50	20
	O ⁶ -MeG ^[b]	0.03	980 ± 118	1000	
dPTP	G	0.05 ± 0.01	160 ± 33	80	
	O ⁶ -MeG	N.D. ^[c]	N.D.		

All values after “±” represent standard deviation from three independent experiments.

^[a] 10³-fold more efficiently incorporated opposite G than O⁶-MeG. Data were consistent with published values,^[9] were standard radiolabeling techniques were used.

^[b] Experiments for 2-thio-dCTP opposite O⁶-MeG were performed in duplicate. All the other steady-state kinetic experiments were performed in triplicate.

^[c] N.D., not detectable. SYBR Gold stained bands corresponding to primer extension product below a limit of detection of 2 nM.