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Structural and kinetic studies of the effect of guanine-N7 alkylation and metal cofactors on DNA replication

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Abstract

A wide variety of endogenous and exogenous alkylating agents attack DNA to preferentially generate N7-alkylguanine (N7-alkylG) adducts. Studies on the effect of N7-alkylG lesions on biological processes have been difficult due in part to complications arising from the chemical lability of the positively charged N7-alkylG, which can readily produce secondary lesions. To assess the effect of bulky N7-alkylG on DNA replication, we prepared chemically stable N7-benzylguanine(N7bnG)-containing DNA and evaluated nucleotide incorporation opposite the lesion by human DNA polymerase β (pol β), a model enzyme for high-fidelity DNA polymerases. Kinetic studies showed that the N7-benzyl-G lesion greatly inhibited dCTP incorporation by pol β . The crystal structure of pol β incorporating dCTP opposite N7bnG showed a Watson-Crick N7bnG:dCTP. The pol β -N7bnG:dCTP structure showed an open protein conformation, a relatively disordered dCTP, and lack of catalytic metal, which explained the inefficient nucleotide incorporation opposite N7bnG. This indicates that pol β is sensitive to major groove adducts in the templating-base side and deters nucleotide incorporation opposite bulky N7-alkylG adducts by adopting a catalytically incompetent conformation. Substituting Mg²⁺ for Mn²⁺ induced an open-to-closed conformational change due to the presence of catalytic metal and stably bound dCTP and increased the catalytic efficiency by ~10-fold, highlighting the effect of binding of incoming nucleotide and catalytic metal on protein conformation and nucleotidyl transfer reaction. Overall, these results suggest that, although bulky alkyl groups at guanine-N7 may not alter base-pairing properties of guanine, the major-groove-positioned lesions in the template could impede nucleotidyl transfer by some DNA polymerases.

Graphical Abstract

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Notes

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Author Contributions

Y.K., M.K., and S.L. conceived and designed the experiments. Y.K. conducted synthetic work and kinetic studies. M.K. performed crystallographic experiments. All authors wrote the manuscript.

Introduction

Endogenous and exogenous alkylating agents attack DNA to produce various alkylated DNA lesions such as O6-, N3-, and N7-alkylpurines, which can affect DNA metabolism.¹ O6-Alkylguanines (e.g., O6-methylguanine) are minor yet highly mutagenic lesions that can cause G to A transition mutations by disrupting Watson-Crick base pairing with cytosine.^{2–3} N3-Alkyladenine lesions do not alter Watson-Crick base pairing but block DNA replication by inhibiting minor groove interactions of DNA polymerases.⁴ N7-Alkylguanine (N7-alkylG) adducts are major lesions both *in vitro* and *in vivo*, but their effects on DNA replication and mutagenesis are poorly understood due in part to the chemical instability of the lesions.⁵

As the most nucleophilic site within the nucleobases of DNA, N7 of guanine is the major site of modification by a large number of alkylating mutagens, carcinogens, and anticancer agents. Over the past few decades, major advances have occurred in understanding of the structural features and/or biological effects of N7-alkylG lesions that are formed by a wide variety of alkylating agents including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, aflatoxin B₁, *o*-quinones, diepoxybutane, ethylene dibromide, leinamycin, and nitrogen mustards (Figure 1C).^{5–10} Small N7-alkylG adducts such as N7-methylguanine (N7mG) are removed by alkyladenine DNA glycosylase (AAG) in humans and AlkA in *E. coli*, whereas bulky N7-alkylG (e.g. aflatoxin B₁-N7G) adducts are preferentially repaired by nucleotide excision DNA repair.^{11–13} If not removed by DNA repair pathways, the positively charged N7-alkylG lesions can undergo spontaneous depurination to produce the highly mutagenic abasic sites, which can cause G to T transversion mutations (Figure 1A).^{14–15} In addition, the imidazole ring of N7-alkylG can be opened at high pH to produce mutagenic alkyl formamidopyrimidine (alkyl-FapyG) lesions (Figure 1A).^{12,16,17}

Despite the advances in understanding of mutagenicity of the secondary lesions derived from N7-alkylG (e.g., abasic sites), the effect of the primary N7-alkylG adducts on DNA replication and mutagenesis remains poorly understood except for few lesions such as N7-G adducts of aflatoxin B₁ and acridine half-mustard ICR-191: the N7-G adducts of aflatoxin B₁ and acridine half-mustard ICR-191 have been shown to induce G to T and G to A mutations, respectively (Figure 1C).^{7,18} The presence of a positive charge at guanine N7 significantly decreases the pK_a of guanine and has been suggested to promote promutagenic base pairing with thymine (Figure 1A).^{19,20} Systematic investigation of the biological effects of N7-alkylG adducts has been hampered due in part to a difficulty in preparation of site-specific incorporated N7-alkylG lesions, which is associated with the instability of the N7-alkylG. We previously solved the instability problem of N7-alkylG adducts by using 2'-

fluorine-mediated transition state destabilization strategy (Figure 1B) and reported the first crystal structure of N7mG base paired with thymine.^{21,22} In duplex DNA, the N7mG:T base pair adopts Watson-Crick-like base pair rather than wobble base pair, supporting that guanine N7 alkylation may promote nucleotide misincorporation by altering base pairing properties of guanine.

To gain insight into the effect of bulky N7-alkylG on DNA replication and mutagenesis, we conducted kinetic and structural studies of human DNA polymerase β (pol β) replicating across templating N7-benzylguanine (N7bnG). We chose N7bnG because *N*-nitrosobenzylmethylamine (NBzMA, Figure 1C), a well-known esophageal carcinogen in laboratory animals, is believed to cause guanine N7-benzylation *in vivo*.^{23–28} In addition, numerous studies have shown the carcinogenic potential of benzylating compounds both *in vivo* and *in vitro*. For example, benzyl halides cause base substitution mutations in *Salmonella typhimurium*, induce sister chromatid exchange in Chinese hamster cells, affect the replication and repair mechanisms in fungi, and induce skin cancers and stomach carcinomas in mice and hepatic and forestomach carcinoma in rats.^{29–33}

For the N7bnG study, we used human DNA polymerase β (pol β) as a model enzyme for high fidelity DNA polymerases.³⁴ Pol β is the smallest mammalian DNA polymerase (39 kD) and plays a critical role in base excision DNA repair pathway by filling short nucleotide gaps. The X-family pol β has medium replication fidelity (10^{-4} to 10^{-5}) due to the lack of a proofreading exonuclease activity.³⁵ However, unlike low fidelity DNA polymerases such as the Y-family DNA polymerase η , pol β undergoes a ligand-induced open-to-closed large conformational change during nucleotide incorporation,^{36,37} which is commonly observed in high fidelity DNA polymerases.^{38,39} In addition, pol β utilizes a strict geometric selection mechanism to increase replication fidelity.^{40–42} In particular, during misincorporation, the enzyme does not readily allow a closed conformation in the presence of Mg^{2+} .⁴¹ In addition to its critical role in base excision DNA repair, pol β has been also implicated in bypass of various types of DNA lesions such as 8-oxoguanine, cisplatin-GG intra-strand cross-link adducts, and UV-induced cyclobutane pyrimidine dimers.^{43–45} In particular, pol β is overexpressed in many cisplatin-treated cancer cells and has been suggested to contribute cisplatin resistance.^{46–48}

In conjunction with our on-going efforts to elucidate the effect of guanine N7-alkylation on DNA replication, herein, we report the synthesis of N7bnG-containing DNA, kinetic results for pol β -catalyzed nucleotide insertion opposite N7bnG, and four crystal structures of pol β incorporating dCTP or dTTP opposite templating N7bnG. Our studies provide insights into the effect of major-groove-positioned bulky lesion on DNA replication.

MATERIALS AND METHODS

Synthesis of 2'-fluoro-2'-deoxy-N7-benzylguanosine (N7bnG) phosphoramidite

Briefly, phenoxyacetyl (Pac) protected 2'-fluoro-2'-deoxyguanosine **2** was prepared from ribose derivative **1** as described previously.²¹ The N2-protected 2'-fluoro-2'-deoxyguanosine **2** was treated with excess benzyl bromide at 25 °C to give 2'-fluoro-2'-deoxy-N7-benzylguanosine **3** in 90% yield (Scheme 1). Dimethoxytritylation of **3**

followed by phosphatidylation provided N7bnG phosphoramidite **5**, which was used for solid phase DNA synthesis.

7-Benzyl-9-(3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-6-oxo-2-(2-phenoxyacetamido)-6,9-dihydro-1H-purin-7-ium (3)—Benzyl bromide (1.8 mL, 15.2 mmol) was added to a suspension of Pac-protected 2'-fluoro-2'-deoxyguanosine **2** (205 mg, 0.76 mmol) in DMF (5 mL). After stirring for 12h at 25 °C, the reaction mixture was poured into Et₂O and the resulting precipitate was collected by filtration and concentrated to afford 246 mg (90%) of N7-benzyl-2'-fluoro-2'-deoxyguanosine **3**. ¹H NMR (400 MHz, DMSO-d₆) δ 10.15 (1H, br, s), 9.70 (1H, s), 9.53 (1H, s), 7.52–7.22 (7H, m), 6.94–6.82 (3H, m), 6.40 (1H, dd, *J* = 3.6, 12.0 Hz), 6.13 (1H, d, *J* = 3.8 Hz), 5.73 (2H, d, *J* = 16 Hz), 5.34 (1H, dt, *J* = 3.6, 52.1 Hz), 5.19 (1H, t, *J* = 6.0 Hz), 4.93 (1H, d, *J* = 9.6 Hz), 4.46 (1H, dd, *J* = 4.0, 12.8 Hz), 4.01 (1H, m), 3.68 (2H, m); HRMS calculated for C₂₅H₂₅FN₅O₆ (M⁺) 510.1784, found 510.1783.

7-Benzyl-9-(5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-3-fluoro-4-hydroxytetrahydrofuran-2-yl)-6-oxo-2-(2-phenoxyacetamido)-6,9-dihydro-1H-purin-7-ium (4)—To a pyridine solution of N7-benzyl-2'-fluoro-2'-deoxyguanosine **3** (125 mg, 0.25 mmol) was added 4,4'-dimethoxytrityl chloride (125 mg, 0.37 mmol). The mixture was stirred for 2h at room temperature, diluted with dichloromethane, and washed with saturated NaHCO₃ and brine. After drying over anhydrous sodium sulfate, the organic layer was filtered, concentrated and purified by silica gel chromatography to give 161 mg (80%) of tritylated compound **4**. ¹H NMR (400 MHz, DMSO-d₆) δ 11.95 (1H, br, s), 9.90 (1H, s), 9.34 (1H, s), 7.50–7.14 (14H, m), 6.98–6.76 (7H, m), 6.43 (1H, dd, *J* = 4.0, 12.4 Hz), 6.06 (1H, d, *J* = 4.2 Hz), 5.69 (2H, d, *J* = 9.2 Hz), 5.33 (1H, dt, *J* = 3.2, 52.4 Hz), 4.97 (1H, d, *J* = 8.8 Hz), 4.59 (1H, dd, *J* = 4.6, 16.8 Hz), 4.02 (1H, m), 3.72 (2H, m); HRMS calculated for C₄₆H₄₃FN₅O₆ (M⁺) 812.3109, found 812.3090.

7-Benzyl-9-(5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-((2-cyanoethoxy)(diisopropylamino)phosphino)oxy)-3-fluorotetrahydrofuran-2-yl)-6-oxo-2-(2-phenoxyacetamido)-6,9-dihydro-1H-purin-7-ium (5)—To a CH₂Cl₂ solution of tritylated compound **4** (125 mg, 0.15 mmol) was added 4,5-dicyanoimidazole (12.7 mg, 0.11 mmole) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphorodiamidite (85.2 μL, 0.3 mmol). After stirring for 1h at 25 °C, the reaction mixture was concentrated and submitted to a silica gel chromatography (+0.5% triethylamine) to afford 120.4 mg (78%) of N7bnG phosphoramidite **5**. ³¹P NMR (400 MHz, CD₃CN) δ 150.67, 150.37; HRMS calculated for C₅₅H₆₀FN₇O₉P (M⁺) 1012.4169, found 1012.4165.

DNA sequences used for kinetic and X-ray crystallographic studies

Oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX) and Integrated DNA Technologies (Coralville, IA). They were purified by the manufacturers and confirmed by MALDI-TOF mass spectrometry. The sequences used for kinetic assays were 5'-FAM/GGGGGCTCGTAAGGATTC-3' for the upstream primer, 5'-phosphate/AGTCGG-3' for the downstream primer, and 5'-CCGACT(X)GAATCCTTACGAGCCCCC-3' [X = dG (control), 2'-fluoro-2'-

deoxyguanine (FdG) or N7bnG] for the template. The DNA sequences used for co-crystallization were 5'-CCGAC(N7bnG)TCGCATCAGC-3' for template, 5'-GCTGATGCGA-3' for the upstream primer and 5'-phosphate/GTCGG-3' for the downstream primer.⁴³ The template oligonucleotide was prepared via solid-phase DNA synthesis using ultra-mild deprotection conditions (50 mM K₂CO₃ in MeOH, 25 °C, 24h). The upstream, downstream primers and template were annealed to give a single-nucleotide gapped DNA, which was used for kinetics and the crystallization of both binary and ternary polβ complex structures.

Expression and purification of human DNA polymerase β

Polβ used for kinetic and crystallographic studies was expressed and purified from *E. coli* as described previously.^{37,41}

Determination of melting temperatures and thermodynamic parameters

Melting temperatures (T_m) for dG:dC-, FdG:dC- (control), N7methyl-dG:dC-, or N7-benzyl-dG:dC-containing 16-mer duplex DNA (5'-CCGACXTCGCATCAGC-3', X = dG, FdG, N7mG, N7bnG; 5'-GCTGATGCG ACGTCGG-3') were determined by using fluorescence measurement that involves a double-stranded DNA-specific dye SYBR Green I. DNA melting curves were acquired using a microvolume fluorometer integrated with a thermal cycler from 7 independent measurements. Thermodynamic parameters were obtained assuming the two-state model according to the relationship: $-\ln K_a = -\ln[2f/C_T(1-f)^2] = H^\circ/RT - S^\circ/R$ (K_a , annealing constants; f, fraction of hybridized duplex; C_T , initial total conc. of ss DNA). The K_a values were calculated at each temperature, and were least-squared fitted to the Van't Hoff relationship. H° and S° values were estimated from slopes and intercepts of fitted straight lines of $\ln K_a$ vs. $1/T$ plots.

Steady-state kinetics of nucleotide incorporation opposite N7bnG by polβ

DNA substrates containing a single-nucleotide gap opposite the templating N7bnG were prepared by annealing the template oligonucleotide (5'-CCGACT(X)GAATCCTTACGAGCCCC-3'; X = N7bnG) with the upstream (5'-FAM/GGGGGCTCGTAAGGATTC-3') and the downstream primers (5'-phosphate/AGTCGG-3') at 95 °C for 3 min followed by slow cooling to 22 °C. Polβ activities were determined using a mixture containing 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 100 mM KCl, 80 nM substrate DNA and varying concentration of the incoming nucleotide. The nucleotidyl transfer reactions were initiated by adding polβ to the mixture. After incubation at 37 °C for 2 min, the reaction was quenched by adding a stop solution containing 95% formamide, 45 mM Tris-borate pH 7.5, 20 mM ethylenediaminetetraacetic acid, 0.1% bromophenol blue and 0.1% xylene cyanol. The nucleotidyl transfer reaction products were resolved on 20% polyacrylamide urea gels, and the product formation was analyzed using a PhosphorImager (GE). The efficiency of nucleotide insertion was calculated as k_{cat}/K_m . The relative efficiency of dNTPs incorporation opposite N7bnG was determined as $f = (k_{cat}/K_m)_{[dNTP:N7bnG]} / (k_{cat}/K_m)_{[dNTP:dG]}$.

Co-crystallization and structure determination of pol β -DNA binary and ternary complexes

The ternary pol β complexes with non-hydrolyzable dCMPNPP or dTMPNPP (Jena Bioscience) in the presence of 4 mM MgCl₂ or MnCl₂ with templating N7bnG were crystallized using the similar conditions described previously.⁴⁹ Diffraction data were collected at 100 K at the beamline 5.0.3 at the Advanced Light Source, Lawrence Berkeley National Laboratory. All diffraction data were processed using HKL2000. Structures were solved by molecular replacement using a gapped binary complex structure with an open conformation (PDB ID: 3ISB) and a ternary complex structure with a closed conformation (PDB ID: 2FMS) as the search models.⁴² The model was built using COOT and refined using Phenix.^{50,51}

RESULTS

The impact of guanine N7 alkylation on the stability of duplex DNA

To evaluate the effect of guanine N7-alkylation on the stability of duplex DNA, we determined melting temperatures (T_m) for dG:dC-, 2'-FdG:dC- (control), N7mG:dC-, or N7bnG:dC-containing 16-mer duplex DNA using fluorescence measurement that involves a double-stranded DNA-specific dye SYBR Green I. We converted DNA fluorescence data into melting peaks after removal of background fluorescence, and determined T_m using a plot of the negative derivative of fluorescence ($-dF/dT$) vs. temperature. Melting temperatures for dG-, 2'-FdG-, N7mG-, and N7bnG-containing 16-mer DNA were 65.4, 65.3, 64.3, and 66.6 °C, respectively (Figure 2 and Table 1). Fluorination at the 2' position did not significantly affect DNA stability and N7-methyl group slightly decreased duplex DNA stability, whereas N7-benzyl group increased the stability. Thermodynamic parameters for thermal denaturation were obtained using Van't Hoff relationship. The enthalpy (ΔH°) of duplex formation was more favorable for the N7mG:dC DNA relative to the dG:dC DNA, but entropy (ΔS°) change was less favorable for the N7mG:dC DNA, thereby slightly destabilizing duplex DNA. Replacing N7-methyl group with N7-benzyl group resulted in favorable enthalpy and entropy changes for duplex formation, thereby stabilizing duplex DNA. The increased stability might be caused by potential hydrophobic interactions (e.g. edge-to-face interaction) between benzyl group and neighboring base(s).

Templating N7bnG greatly deters nucleotide incorporation by pol β

To assess whether the major-groove-positioned N7-benzyl group affects polymerase activity, we determined kinetic parameters for pol β -catalyzed insertion of dCTP and dTTP opposite templating N7bnG (Table 2).⁴² Fluorination at the 2' position has been shown to exert a minimal effect on the catalysis of DNA polymerases (Table 2).^{49,52} In the presence of Mg²⁺, the catalytic efficiency for dCTP incorporation opposite N7bnG decreased ~6000 fold compared to a templating dG, indicating that bulky N7-benzyl group greatly inhibited nucleotide incorporation opposite the lesion. Substituting Mg²⁺ for Mn²⁺ increased the dCTP insertion efficiency ~10 fold, highlighting the effect of metal ion on the catalytic efficiency. In addition to the N7bnG:dCTP kinetics, we examined the effect of guanine N7-benylation on the formation of G:T mismatches, which comprise about 60% of pol β -induced spontaneous base substitutions.⁵³ The templating N7bnG almost completely inhibited dTTP incorporation by the enzyme. Overall, the steady-state kinetic studies

indicated that templating N7bnG greatly deterred the incorporation of nucleotides by a medium-fidelity DNA polymerase. These results suggest that bulky N7-alkylguanine lesions at templating position prevents nucleotide incorporation by some DNA polymerases.

Pol β adopts a catalytically incompetent conformation in the presence of Watson-Crick N7bnG:dCTP base pair

The major groove N7 benzyl group neither blocks minor groove interaction nor prevents Watson-Crick base pairing. How does a templating N7bnG strongly inhibit dCTP incorporation by pol β ? To provide structural basis for the inefficient insertion of dCTP opposite N7bnG (Table 2), we solved ternary structure of pol β with templating N7bnG base-paired with an incoming non-hydrolyzable dCTP analog dCMPNPP (dCTP* hereafter) in the presence of Mg²⁺. The non-hydrolyzable nucleotide analog, which can be coordinated to the active site with a conformation essentially identical to that of the natural dNTP, has been used in many DNA polymerase structures.^{42,54–56} The pol β -N7bnG:dCTP*-Mg²⁺ ternary complex structure was refined to 2.6 Å resolution and solved by molecular replacement (Table 3).

The N7bnG:dCTP*-Mg²⁺ structure clearly explains why the dCTP insertion opposite N7bnG is strongly inhibited (Figure 3). A typical pol β structure with a correct base pairing shows a closed protein conformation. In contrast, the N7bnG:dCTP* structure showed an open protein conformation, indicating that the bulky lesion affected protein conformation. The overall conformation of N7bnG:dCTP* structure was largely different from that of the published pol β -dA:dUTP*-Mg²⁺ structure (RMSD = 2.115 Å).⁴² The α -helix N, which contains minor groove-interacting amino acid residues Asn279 and Arg283, was in an open conformation and is ~7 Å away from the position observed in the closed conformation (Figure 3B). In addition to the altered protein conformation, the catalytic metal ion, which is critical for nucleotidyl transfer reaction, was not observed in the N7bnG:dCTP* structure (Figure 3B). Although the nucleophilic 3'-OH of primer terminus was proximal to P $_{\alpha}$ of incoming dCTP*, the absence of the catalytic metal ion made the enzyme in a catalytically incompetent conformation. Furthermore, the nucleotide-binding Mg²⁺ had only 3 ligands with distances of 2.2, 2.3 and 3.3 Å. Asp192, which typically is liganded to nucleotide-binding metal ion, is ~6 Å away from the metal B. The incomplete coordination with Mg²⁺ at the metal B site would greatly slow nucleotide incorporation opposite the lesion. The weak electron density for the sugar moiety of dCTP* suggests the formation of multiple conformations, which would weaken base pairing interactions with templating N7bnG (Figure 3C). Although dCTP* forms Watson-Crick pair with N7bn, the lack of the catalytic metal ion coordination appears to make dCTP* shift away from the 3' primer terminus, rotating the cytosine nucleobase ~2 Å away from the position observed in the dA:dUTP structure (Figure 3D). Due to the open conformation of α -helix N, minor groove interactions of DNA with Asn279 or Arg283 are lacking. In addition, Tyr271 is H-bonded to templating N7bnG rather than the primer terminus.

To evaluate whether the 2'-fluorination induced the open protein conformation of the pol β -N7bnG:dCTP* complex, we also determined cocrystal structure of pol β in complex with FdG:dCTP* base pair. The FdG:dCTP* structure showed a closed protein conformation and

was essentially identical to the dA:dUTP* structure (Figure 3E), indicating that the 2'-fluorination did not significantly influence the protein conformation and the N7-benzyl moiety induced an open protein conformation.

The pol β -N7bnG:dCTP-Mg²⁺ structure with an open conformation underscores that pol β is sensitive to the presence of an abnormal base at templating position. Although N7bnG disrupts neither Watson-Crick base pairing nor minor groove interaction, the enzyme appears to sense an aberration of templating base within the active site architecture, thereby preventing the formation of a catalytically competent protein conformation.

Comparison of the N7bnG:dCTP* structure with the published pol β structure with N7mG:dCTP* base pair (PDB ID 4O5K, RMSD = 1.482 Å) reveals the effect of steric bulkiness of N7-alkyl group on the conformation of DNA polymerase (Figure 3F).⁴⁹ When N7mG adduct is at the templating position, pol β adopts a closed conformation, showing that small alkyl group at the N7 position of G minimally affect pol β conformation. The pol β 's conformational difference between the N7bnG:dCTP* and N7mG:dCTP* structures suggests that the steric bulkiness of N7-alkylG adducts may significantly affect the conformational reorganization of some DNA polymerases. Taken together, the N7bnG:dCTP* structure indicates that, even when paired with a correct incoming nucleotide, bulky N7-benzyl group at the templating position can alter conformation of pol β and the coordination of catalytic metal ion, thereby impeding the incorporation of dCTP opposite the lesion.

Binding of catalytic metal and incoming nucleotide in stable orientation induces an open-to-closed conformational change in the presence of N7bnG:dCTP base pair

The N7bnG:dCTP-Mg²⁺ complex with an open protein conformation and one metal ion does not represent a catalytically competent conformation. The absence of a metal ion in the catalytic site would significantly slow insertion, which is consistent with the greatly reduced catalytic efficiency for insertion opposite N7bnG. In the N7bnG:dCTP-Mg²⁺ complex, the population of the catalytically competent conformation appears to be much lower than that of incompetent conformation. Our kinetic studies showed that substitution of the active site Mg²⁺ for Mn²⁺ increased the enzyme's efficiency of dCTP insertion opposite N7bnG by 10-fold (Table 2). The result suggests that the Mn²⁺-bound structure may adopt a catalytically more favorable conformation than the Mg²⁺-bound structure and may provide structural basis for the catalytically competent conformation for dCTP insertion opposite N7bnG by pol β . To gain insights into the increased insertion efficiency and catalytically competent conformation, we determined ternary structure of pol β -N7bnG:dCTP* in the presence of Mn²⁺. The pol β -N7bnG:dCTP*-Mn²⁺ complex structure (PDB ID 4YMO) was refined to 2.2 Å resolution (Figure 4).

The overall structure of the pol β -N7bnG:dCTP*-Mn²⁺ complex was significantly different from that of the pol β -N7bnG:dCTP*-Mg²⁺ complex (RMSD = 1.393 Å) (Figure 4). Unlike the Mg²⁺-bound structure, the Mn²⁺-bound structure showed a closed protein conformation (Figures 4A and 4B). In addition, the structure showed the presence of catalytic metal ion, which was coordinated to the 3'-OH of the primer terminus, three catalytic aspartates and α -phosphate oxygen (Figure 4B). In the Mg²⁺-bound complex, the incoming dCTP was

disordered (B-factor values > 70), had poor electron density and was not bound in the catalytically competent conformation (Figure 3C). On the contrary, in the Mn^{2+} -bound complex, the incoming dCTP was ordered (B-factor values ~10–20), had clear electron density and was bound in the catalytically competent conformation (Figure 4C). These structural differences indicate that Mn^{2+} promotes a stable binding of dCTP, which in turn facilitates an open-to-closed conformational change of protein.

The nascent N7bnG:dCTP base pair is now sandwiched between the primer terminus base pair and α -helix N. The minor groove recognizing amino acid residues Asn279, Arg283 and Tyr271 engaged in hydrogen bonds with the minor groove edges of incoming nucleotide, templating N7bnG, and primer terminus, respectively. Unlike the Mg^{2+} -bound structure, the electron density for the sugar moiety of dCTP* was strong, indicating base pairing interactions with templating N7bnG. The open-to-closed conformational reorganization of protein induced ~2 Å shift of dCTP toward the primer terminus. In the Mg^{2+} -bound structure, N7bnG did not make any interactions with protein. In Mn^{2+} -bound structure, the benzyl moiety of N7bnG was stacked between Lys280 in α -helix N and Asn37 in the lyase domain (Figure 4D). This stacking interactions would further stabilize a closed conformation and promote catalysis. Taken together, the conformation of the Mn^{2+} -bound structure is catalytically more favorable than that of the Mg^{2+} -bound structure, which is consistent with the Mn^{2+} -induced increase in the catalytic efficiency (Table 2). The results highlight the effect of the catalytic metal ion on protein conformation and catalytic efficiency.

Pol β prevents dTTP incorporation opposite templating N7bnG by inducing a staggered base pair and adopting an open conformation

As described in the kinetic studies (Table 2), we did not observe incorporation of dTTP opposite templating N7bnG under varying conditions such as higher concentrations of protein and dTTP and extended reaction times. To gain insight into the observation, we solved structure of pol β in complex with N7bnG:dTTP* in the presence of Mn^{2+} . The dTTP*- Mn^{2+} structure was refined to 2.3 Å resolution.

The N7bnG:dTTP*- Mn^{2+} structure (PDB ID 4YN4) shows a catalytically incompetent conformation (Figure 5A). Protein adopted an open conformation, where α -helix N was about 10 Å away from templating N7bnG (Figure 5B). In addition, the nascent N7bnG:dTTP* base pair assumed a staggered conformation and did not form any interbase hydrogen bonds (Figure 5B). The catalytic Asp256 was not coordinated to the catalytic metal ion. In the structure, the nucleobase of dTTP* and the benzyl moiety of N7bnG were disordered, as indicated by weak electron density (Figure 5C).

The dTTP*- Mn^{2+} structure was largely different from the dCTP*- Mn^{2+} structure (Figure 5D), indicating that the enzyme strongly discriminated between correct and incorrect incoming nucleotides in the active site. When templating N7bnG was paired with incorrect incoming nucleotide, pol β did not allow a closed conformation even in the presence of the promutagenic Mn^{2+} .

Overall structure of the dTTP*-Mn²⁺ complex was very similar to that of the dCTP*-Mg²⁺ complex (RMSD = 0.433 Å, Figure 5E). Conformations of template, upstream and downstream primers and protein between the dTTP*-Mn²⁺ and dCTP*-Mg²⁺ structures were essentially identical. The only notable difference is found in the conformation of incoming nucleotide. While protein in the both dTTP*-Mn²⁺ and dCTP*-Mg²⁺ structures adopted an open conformation, only dCTP formed a coplanar pair with N7bnG. The dTTP*-Mn²⁺ structure with an open conformation and a staggered base pair conformation indicates that polβ strongly deters the incorporation of dTTP opposite templating N7bnG, which is in consistent with the lack of dTTP incorporation opposite N7bnG by the enzyme.

Comparison of the N7bnG:dTTP*-Mn²⁺ structure with published dG:dTTP*-Mn²⁺ structure (PDB ID 4PGX) reveals the effect of N7-alkylation on the conformation of mismatched base pairs (Figure 5F).⁴¹ The published dG:dTTP*-Mn²⁺ structure shows that dG:dTTP forms Watson-Crick-like base pair conformation and polβ adopts a closed conformation. This is in contrast to the N7bnG:dTTP* structure with a staggered base pair conformation and an open conformation. Watson-Crick-like dG:dTTP can occur through either ionization or tautomerization.^{41,57} In addition, in the absence of protein contact, N7-methylation has been shown to induce Watson-Crick-like N7mG:T base pair, which presumably occurs via either ionization or tautomerization of N7mG.²² It appears that the active site architecture of polβ deters the formation of Watson-Crick-like N7-alkylG:T base pair while allowing Watson-Crick-like G:T base pair.

DISCUSSION

Implication of Watson-Crick N7bnG:dCTP base pair with an open DNA polymerase conformation

The polβ-N7bnG:dCTP-Mg²⁺ structure with an open conformation highlights the effect of the bulky N7-alkylG adduct at templating position on the conformation and catalytic efficiency of DNA polymerases. An open polβ conformation and Watson-Crick dG:dCTP base pair is observed in structure with an Arg283Lys mutation, which disrupts a critical water-mediated hydrogen bond between Arg283 and Glu295 that is required for the formation of a closed polβ conformation.⁵⁸ The Arg283Lys polβ-dG:dCTP structure with an open protein conformation is consistent with a drastic decrease in catalytic efficiency. Unlike the polβ-N7bnG:dCTP-Mg²⁺ structure, the published polβ structure with N7mG:dCTP and active site Mg²⁺ shows a closed protein conformation.⁴⁹ The conformational difference between the N7bnG and the N7mG structures indicates that steric bulkiness of the N7-alkyl group at templating base greatly affects protein conformation. Recent studies with incoming therapeutic nucleotides show that major groove modification at an incoming nucleotide is well accommodated in the catalytic site of polβ.⁵⁹ This difference appears to be caused by differences between the templating base and incoming nucleotide major groove adducts. The major groove on templating base side is restricted by the lyase domain, whereas that on incoming-nucleotide side has a cavity to accommodate major groove adducts.

The observation of the ~6,000-fold reduction in catalytic efficiency (Table 2) and N7bnG:dCTP-Mg²⁺ structure with a catalytically incompetent conformation suggests that a bulky N7-alkylG adduct at templating position is a strong block to DNA replication at least

by pol β . The stalled DNA replication fork would be resolved by alkylation damage-specific DNA glycosylases (e.g., *E. coli* AlkA, human alkyladenine DNA glycosylase) and/or nucleotide excision DNA repair. The strong inhibition of nucleotide incorporation opposite N7bnG by pol β implies that the mutagenic potential of bulky N7-alkylG lesions would be low, unless they undergo spontaneous depurination or imidazole ring opening to generate secondary lesions.

The lack of coordination of the catalytic metal (metal A) and unstable binding of dCTP observed in the pol β -N7bnG:dCTP-Mg²⁺ structure suggest that coordination of the catalytic metal and stable binding of incoming nucleotide are critical to nucleotide incorporation and adducts preventing such events abrogate catalytic activity. DNA polymerase structures lacking catalytic metal coordination have been observed in complexes containing dideoxy primer terminus (e.g., ddC), which prevents full coordination of the catalytic metal ion.⁶⁰ In addition, pol β structures with mismatched base pairs (e.g., G:T) or Arg283Lys mutation lack the catalytic metal coordination.^{41,58} Unlike the published structures, pol β -N7bnG:dCTP-Mg²⁺ complex involves neither mutation in DNA polymerase nor dideoxy primer terminus. The pol β structures with one metal ion and an open conformation indicates that binding of incoming nucleotide to the active site occurs prior to the coordination of the catalytic metal ion and the conformational change of protein. The lack of catalytic metal ion coordination and the formation of an open protein conformation are consistent with the dramatic decrease in dCTP insertion opposite N7bnG. The lack of the catalytic Mg²⁺ binding in the presence of templating N7bnG suggests that pol β prevents coordination of the catalytic metal ion in the presence of damaged base pairs. It would be of interest whether other DNA polymerases (e.g., X-family DNA polymerase λ) also prevent the catalytic metal ion coordination in the presence of N7-alkyl guanine lesions.

The effect of metal cofactors and incoming nucleotide on the conformational change of DNA polymerases and base pairs

Although pol β lacks an intrinsic proofreading exonuclease activity, the enzyme increases the replication fidelity by inducing staggered base pairs, generating an abasic site-like intermediate and shifting the templated strand for mismatches.⁵⁴ The catalytic metal-dependent conformational reorganization observed in pol β structures provides insights into the enzyme's strategy to prevent insertion opposite bulky N7-alkylG lesion (Figure 6). Catalytic metal-dependent open-to-closed conformational change of protein has been rarely observed in other DNA polymerase structures. For instance, in the presence of nascent O6mG:dTTP or O6mG:dCTP base pair, the A-family *Bacillus stearothermophilus* DNA polymerase I large fragment adopts a closed conformation when Mg²⁺ was used.³ In addition, the B-family DNA polymerase RB69 adopts a closed conformation in the presence of wobble dG:dTTP and Mg²⁺.⁶¹ We previously reported that the use of Mn²⁺ induced an open-to-closed conformational change of pol β and the formation of Watson-Crick-like O6mG:dTTP and dG:dTTP base pairs.^{41,62} In contrast, pol β complexes containing O6mG:dCTP and N7mG:dTTP base pairs do not undergo a metal-dependent conformational change and show a staggered base pair conformation. The conformational differences among these structures suggest that catalytic metal-dependent conformational change of pol β

readily occurs when a nascent base pair forms Watson-Crick-like conformation within the active site architecture.

Pol β structures with Mn $^{2+}$ -induced closed conformation may represent a close approximation of a catalytically competent state for misincorporation (e.g., dG:dTTP) or insertion opposite a lesion (e.g., N7bnG:dCTP), which would be difficult to capture when Mg $^{2+}$ is used. The Mg $^{2+}$ -bound pol β structure with dG:dTTP mismatch shows an open protein conformation, a staggered base pair conformation, and one active site metal ion, which is catalytically incompetent.⁴¹ The N7bnG:dCTP-Mg $^{2+}$ and dG:dTTP-Mg $^{2+}$ structures with an open conformation suggest that the population of the catalytically competent state in the presence of Mg $^{2+}$ would be low, precluding the capture of the catalytically competent conformation. The use of Mn $^{2+}$, which binds to the catalytic site more tightly than Mg $^{2+}$ does, would facilitate catalytic-metal coordination and stabilize binding of incoming nucleotide to the catalytic site (Figure 6). This would increase the population of a catalytically competent state for nucleotide incorporation, leading to the capture of a catalytically competent conformation.

The Mn $^{2+}$ -mediated conformational change observed in the pol β -N7bnG:dCTP structure is different from that observed in the pol β -O6mG:dTTP and pol β -dG:dTTP structures. N7bnG:dCTP forms a Watson-Crick base pair with a coplanar conformation in the presence of Mg $^{2+}$ whereas O6mG:dTTP and dG:dTTP form a staggered conformation in the presence of Mg $^{2+}$. Therefore, substituting Mg $^{2+}$ for Mn $^{2+}$ induces conformational change of protein only in the case of the N7bnG:dCTP complex, whereas it induces a conformational change of both protein and base pair in the case of O6mG:dTTP and dG:dTTP structures.

The effect of N7 alkylation on base pairing properties of guanine

How does pol β disallow the formation of Watson-Crick-like N7-alkylG:dTTP base pair in the active site? Guanine N7-methylation has been shown to decrease pK_a of the N1 of guanine by ~2 unit: the pK_a of guanine N1 of 7,9-dialkylguanine is ~7, enabling ionization at physiological pH.¹⁹ The pK_a of N7-alkylG would increase the population of zwitterionic form of N7-alkylG, which could promote base pairing with thymine and thus induce G to A transversion mutations. For example, the N7-G adduct of acridine half-mustard preferentially induces G to A mutations.^{63,64} Our recent crystal structure shows that, in duplex DNA, N7mG:dT base pair adopts a Watson-Crick-like geometry.²² The Watson-Crick-like N7mG:dT base pair conformation indicates the involvement of zwitterionic or enol tautomeric form of N7mG when pairing with thymine. However, in the active site of pol β , the N7mG:dTTP and N7bnG:dTTP base pairs take on a staggered conformation even in the presence of Mn $^{2+}$, suggesting that N7mG and N7bnG mainly exist as their keto tautomers in the nascent base pair site. The difference between N7mG:dT and N7-alkylG:dTTP base pair conformations might result from the difference in microenvironment around the base pairs. One possibility is that the formation of zwitterionic or enol tautomeric form of N7-alkylG may require a highly constrained environment, so the middle of duplex DNA might be more favorable for isomerization than the nascent base pair site. Determining whether other DNA polymerases induce Watson-Crick-like N7-alkylG:dTTP base pair in the active site would be of interest.

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Figure 1. Mutagenic effects of N7-alkylG lesions using a 2'-fluorine-mediated transition-state destabilization strategy. (A) Processing of chemically unstable N7-alkylG lesions. N7-alkylG lesions can undergo imidazole-ring opening or depurination to generate promutagenic alkyl-Fapy or abasic sites, respectively. The ionized N7-alkylG could form Watson-Crick-like base pairing with thymine during replication, promoting G to A transition mutations. (B) Inhibition of depurination by 2'-fluorine-mediated transition-state destabilization. (C) Representative alkylating agents that preferentially attack N7 of guanine.

Figure 2. Melting peaks for duplex DNA containing dG:dC, FdG:dC (control), N7mG:dC, or N7bnG:dC. Fluorescence of SYBR Green I as a function of temperature was monitored. Rapid loss of fluorescence occurs at near T_m .

Figure 3.

Ternary structure of polβ in complex with templating N7bnG paired with dCTP* in the presence of the active site Mg²⁺ (PDB ID: 4YMN). (A) Overall view of the polβ-N7bnG:dCTP*-Mg²⁺ structure. DNA template strand is shown in yellow, primer strands are in orange, and polβ is in cyan. (B) Close-up view of the active-site structure with an open protein conformation. (C) The 2F_o-F_c electron density map around N7bnG:dCTP* contoured at 1σ level is shown. Electron density of the sugar moiety of the incoming dCTP* is weak. (D) Comparison of the active site of the polβ-N7bnG:dCTP*-Mg²⁺ structure (in yellow, PDB ID: 4YMN) with that of the published structure with correct insertion (PDB ID: 2FMS (21)). (E) Comparison of polβ-FdG:dCTP* structure (green, PDB ID: 5EOZ) with the published structure with correct insertion (yellow, PDB ID: 2FMS²¹). In the FdG:dCTP structure, the coordination distance, ligand number and lack of coordination of the 3'-OH to the catalytic metal indicated binding of Na⁺ to the metal B site. The Na⁺ is shown in blue sphere. (F) Comparison of the active site of the polβ-N7bnG:dCTP*-Mg²⁺ structure (blue) with the published polβ-N7mG:dCTP*-Mg²⁺ structure (orange, PDB ID: 4O5K⁴⁹).

Figure 4.

Ternary structure of polβ in complex with N7bnG paired with dCTP* in the presence of Mn²⁺. (A) Overall structure of the polβ-N7bnG:dCTP*-Mn²⁺ complex. (B) Close-up view of the active site of polβ-N7bnG:dCTP*-Mn²⁺ structure. Note that α-helix N is in closed conformation and Mn²⁺ coordinates with the three catalytic aspartates, 3'-OH of primer terminus and the Pα of the incoming dCTP*. (C) 2F_o-F_c electron density map around N7bnG:dCTP* contoured at 1σ level is shown. Unlike the Mg²⁺-bound structure, the Mn²⁺-bound structure shows strong electron density for the sugar moiety of dCTP*, indicating that the incoming nucleotide is stabilized within the active site architecture. (D) Stacking interaction of N7bnG with Asn37 and Lys280. Van der Waals radii of the benzyl moiety and the side chains of Asn37 and Lys280 are shown in spheres. (E) Comparison of the active site of N7bnG:dCTP*-Mn²⁺ structure (blue) with that of the N7bnG:dCTP*-Mg²⁺ structure (yellow). Note differences in α-helix N conformation, the nascent base pair conformation and the active site metal ions.

Figure 5.

Ternary structure of polβ in complex with templating N7bnG paired with an incoming dTTP* in the presence of Mn²⁺ (PDB ID: 4YN4). (A) An overall view of the polβ-N7bnG:dTTP*-Mn²⁺ structure. N7bnG is shown in purple and incoming dTTP* is shown in blue. (B) A close-up view of the active site of polβ-N7bnG:dTTP*-Mn²⁺ structure. N7bnG:dTTP base pair forms a staggered conformation. (C) 2F_o-F_c electron density map around N7bnG:dTTP* base pair contoured at 1σ level. (D) Comparison of the N7bnG:dTTP*-Mn²⁺ structure with the N7bnG:dCTP*-Mn²⁺ structure. (E) Comparison of the N7bnG:dTTP*-Mn²⁺ structure with the N7bnG:dCTP*-Mg²⁺ structure. (F) Comparison of the N7bnG:dTTP*-Mn²⁺ structure with published polβ-dG:dTTP*-Mn²⁺ structure (PDB ID 4PGX (41)).

Figure 6.

The catalytic-metal coordination-induced conformational change of the polβ-N7bnG:dCTP complex. In the Mg²⁺-bound structure, the catalytic metal was lacking; dCTP was unstably bound; and polβ adopts an open conformation. In the Mn²⁺-bound structure, the catalytic metal was present; dCTP was stably bound; Bn group involved in stacking interaction; and polβ was in a closed conformation. In both structures, N7bnG:dCTP formed Watson-Crick base pair with three interbase hydrogen bonds. The benzyl moiety is shown in magenta and metal ions are shown in spheres. The α-helix N of polβ is colored in red.

Scheme 1.

Synthesis of N7-benzylguanine-containing DNA **6** starting from commercially available ribose derivative **1**. N7-benzyl-2'-fluoro-2'-deoxyguanosine phosphoramidite **5** was prepared from N2-phenoxyacetyl-2'-fluoro-2'-deoxyguanosine **2** (21) and incorporated into DNA via solid phase DNA synthesis. (a) Benzyl bromide (20 equiv.), DMF, 25 °C 12h, 90%. (b) DMTrCl (1.3 equiv.), pyridine, 25 °C 2h, 80%. (c) (*i*-Pr₂N)₂P(OC₂H₄CN), 4,5-dicyanodimidazole, CH₂Cl₂, 25 °C 1h, 78%. (d) Solid phase DNA synthesis and ultra-mild deprotection conditions (K₂CO₃, MeOH, 25 °C).

Table 1

Melting temperature (T_m) and thermodynamic parameters for denaturation of double strand DNA containing dG, FdG, N7-methylG (N7mG), and N7-benzylG (N7bnG). T_m , thermodynamic parameters and their standard deviations are an average of 7 independent determinations.

	T_m	G°	H°	$T S^\circ$
dG:dC	65.4 (± 0.04)	-50.0 (± 0.6)	-91.1 (± 0.7)	-41.1 (± 2.0)
FdG:dC	65.3 (± 0.03)	-49.1 (± 1.1)	-90.5 (± 1.2)	-41.4 (± 2.0)
N7mG:dC	64.3 (± 0.06)	-45.7 (± 1.1)	-93.6 (± 1.2)	-47.9 (± 1.4)
N7bnG:dC	66.6 (± 0.07)	-53.6 (± 1.0)	-96.8 (± 1.3)	-43.2 (± 2.4)

Table 2

Kinetics of single nucleotide insertion opposite templating N7bnG by polβ

template:dNTP	metal ion	K_m (μM)	k_{cat} ($10^{-3}s^{-1}$)	k_{cat}/K_m ($10^{-3}s^{-1}μM^{-1}$)	f^a
dG:dCTP ^b	Mg ²⁺	0.59 ± 0.03	20.38 ± 0.50	34.54	1
FdG:dCTP ^b	Mg ²⁺	0.30 ± 0.07	12.52 ± 0.53	36.82	1.1
N7bnG:dCTP	Mg ²⁺	20.24 ± 0.06	0.11 ± 0.02	0.0054	1.6×10 ⁻⁴
N7bnG:dCTP	Mn ²⁺	6.15 ± 0.09	0.32 ± 0.03	0.052	1.5×10 ⁻³
N7bnG:dTTP	Mg ²⁺	-	-	-	-

^aRelative efficiency:(k_{cat}/K_m)[dNTP:N7bnG]/(k_{cat}/K_m)[dCTP:dG];

^bReference 49

Table 3

Data collection and refinement statistics

PDB CODE	FdG:dCTP Mg ²⁺ (5EOZ)	N7bnG:dCTP Mg ²⁺ (4YMN)	N7bnG:dCTP Mn ²⁺ (4YMO)	N7bnG:dTTP Mn ²⁺ (4YN4)
Data Collection				
space group	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁
Cell Constants				
a (Å)	50.754	54.907	50.728	54.774
b	80.015	79.020	79.170	79.179
c	55.589	55.019	55.634	55.031
α (°)	90.00	90.00	90.00	90.00
β	107.77	107.92	107.24	106.53
γ	90.00	90.00	90.00	90.00
resolution (Å) ^a	20–2.09 (2.13–2.09)	20–2.58 (2.62–2.58)	20–2.15 (2.19–2.15)	20–2.25 (2.29–2.25)
R _{merge} ^b (%)	0.098 (0.447)	0.110 (0.545)	0.108 (0.425)	0.081 (0.411)
<I/σ>	18.1 (2.70)	15.7 (2.48)	14.2 (2.12)	21.6 (2.68)
completeness (%)	100.0 (100.0)	100.0 (99.7)	100.0 (99.8)	99.9 (98.8)
redundancy	4.7 (4.1)	5.6 (5.2)	4.7 (4.1)	5.5 (5.2)
Refinement				
R _{work} ^c /R _{free} ^d (%)	18.3/23.8	20.3/26.9	19.6/22.6	20.5/25.5
unique reflections	25149	13902	22917	21437
Mean B Factor				
(Å ²)				
protein	26.46	41.40	24.69	37.36
ligand	14.47	34.62	35.36	36.77
solvent	28.64	34.93	27.95	33.48
Ramachandran				
Plot				
most favored (%)	97.5	95.0	97.8	94.4
add. allowed (%)	2.5	5.0	2.2	5.6
RMSD				
bond lengths (Å)	0.009	0.009	0.006	0.007
bond angles (degree)	1.030	1.618	1.471	1.487

^aValues in parentheses are for the highest resolution shell.^b $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$ where I is the integrated intensity of a given reflection.^c $R_{\text{work}} = \sum |F(\text{obs}) - F(\text{calc})| / \sum F(\text{obs})$.^d $R_{\text{free}} = \sum |F(\text{obs}) - F(\text{calc})| / \sum F(\text{obs})$, calculated using 5% of the data.