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Steric and Electrostatic Effects at the C2 Atom Substituent Influence Replication and Miscoding of the DNA Deamination Product Deoxyxanthosine and Analogs by DNA Polymerases

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Abstract

Deoxyinosine (dI) and deoxyxanthosine (dX) are both formed in DNA at appreciable levels *in vivo* by deamination of deoxyadenosine (dA) and deoxyguanosine (dG), respectively, and can miscode. Structure-activity relationships for dA pairing have been examined extensively using analogs but relatively few studies have probed the roles of the individual hydrogen-bonding atoms of dG in DNA replication. The replicative bacteriophage T7 DNA polymerase/exonuclease[−] (pol T7[−]) and the translesion DNA polymerase *Sulfolobus solfataricus* pol IV (Dpo4) were used as models to discern the mechanisms of miscoding by DNA polymerases. Removal of the 2-amino group from the template dG (i.e., dI) had little impact on the catalytic efficiency of either polymerase, as judged by either steady-state or pre-steady-state kinetic analysis, although the misincorporation frequency was increased by an order of magnitude. dX was highly miscoding with both polymerases, and incorporation of several bases was observed. The addition of an electronegative fluorine atom at the 2-position of dI lowered the oligonucleotide T_m and strongly inhibited incorporation of dCTP. The

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Supplementary Data

Supplementary data associated with this article can be found in the on-line version. Supplementary material includes derivation of equations and supplementary Tables S1-S6 and Figs. S1-S12 showing CID fragmentation of oligonucleotide reaction products, steady-state kinetic parameters for next base extension from dG or modified dG:dN template primer termini by pol T7[−] and Dpo4, characterization of the 36-mer oligonucleotide containing 2-BrdI, analysis of the oligonucleotide containing 2-FdI after incubation with 50 mM Tris-HCl buffer, pre-steady-state kinetics of nucleotide incorporation of dCTP opposite dI or 2-FdI in MOPS buffer by pol T7[−], estimation of k_{pol} and $K_d, dCTP$ for pol T7[−] and Dpo4 by pre-steady-state burst rate dependence on dCTP concentration, pre-steady-state and steady-state kinetics of misincorporation by pol T7[−], steady-state kinetics of misincorporation of dTTP opposite dG, dI, 2-FdI, or 2-BrdI by Dpo4, CD spectra of oligonucleotides containing dG, dI, 2-FdI, and 2-BrdI (36-mers) hybridized to an 11-mer overlapping the dG or substituted base, T_m estimates of double-stranded DNA containing dG or dI, 2-FdI, or 2-BrdI in the same position of the template strand, and determination of pK_a by pH titrations of dG, dI, 2-FdI, 2-BrdI, and dX using UV spectra.

addition of bromine or oxygen (dX) at C2 lowered the T_m further, strongly inhibited both polymerases, and increased the frequency of misincorporation. Linear activity models show the effects of oxygen (dX) and the halogens at C2 on both DNA polymerases as mainly due to a combination of both steric and electrostatic factors, producing a clash with the paired cytosine O2 atom, as opposed to either bulk or perturbation of purine ring electron density alone.

Keywords

DNA polymerase; translesion DNA synthesis; DNA deamination; DNA fidelity; steady-state enzyme kinetics

Introduction

The preservation and transmittal of accurate genetic information is critically dependent upon the fidelity of DNA replication.¹ Most DNA polymerases are highly accurate, and > 100 human genes are known to be involved in DNA repair, emphasizing the importance of genetic integrity in biological systems. Even with the presence of all of these DNA repair systems, a finite level of DNA modification exists and challenges the DNA polymerases.² The issue of exactly what drives the high fidelity of DNA replication has been the subject of considerable interest for the past 55 years. The roles of “Watson-Crick” hydrogen bonds have been recognized since the elucidation of the structure of DNA,³ but energetic considerations argue that the specificity of dG:dC and dA:dT pairing is not sufficient to account for the selectivity.⁴ Moreover, fidelity varies considerably among DNA polymerases. One view is that DNA polymerases have steric restraints and “search” for pairs that have the overall molecular dimensions of dG:dC and dA:dT pairs.^{5,6} Consistent with this hypothesis are observations with isosteric pairs of bases, where polymerization activity is observed.^{2,7-14} With such base isosteres there is evidence that stacking forces are involved, arguing that these and other forces may also be at work with the four canonical bases.²

Oxidative deamination of the purine bases is known to occur during inflammation and other biological processes, e.g. with nitric oxide.¹⁵⁻¹⁷ This process converts dA to dI and dG to dX, respectively (Fig. 1), and can change the coding properties at individual bases in the genome.^{15,18-20} Levels of ~5 dX molecules (measured as the base hypoxanthine) per 10⁷ nucleotides have been detected in mouse tissues (a level intermediate between those found for the well-studied 7,8-dihydro-8-oxo dG and the major malondialdehyde-dG adduct).²¹ Treatment of TK6 human lymphoblast cells with nitric oxide led to a 47-fold increase in DNA hypoxanthine (i.e., dX) and 15- to 18-fold increases in *hprt* and *tk6* mutations.¹⁶ Nitric oxide is produced during inflammation, in host defense,¹⁸ and also thermal injury to tissues.²² The mutations generated by treatment with nitric oxide were predominantly dA:dT to dG:dC and dG:dC to dA:dT transitions,²³ being consistent with what is known about *in vitro* miscoding properties of dI and dX, respectively, in some systems.^{19,20} The biological relevance of dX also has some support in the existence of a repair pathway system, *Escherichia coli* endonuclease V.²⁴

Yasui *et al.* 20 recently reported that human pol α , pol η , and a shortened version of pol κ all inserted predominantly dCTP opposite dI, and the corresponding dA:dT to dG:dC transition was seen exclusively in a site-specific mutagenesis study with a c-Ha-ras gene in a mammalian cell system.²⁵ The same group¹⁹ also showed that these three human polymerases incorporated dTTP opposite dX, but pol β preferentially incorporated dCTP. Pol α paired dCTP and dTTP opposite dX in other studies.^{26,27} Wuenschell *et al.*²⁸ reported that *E. coli* DNA polymerase I (Klenow fragment) incorporated dCTP opposite dX (see also ²⁹) but that HIV-1 reverse transcriptase inserted dTTP at a frequency one-fourth that of dCTP. The molecular basis for the effects on these polymerases is still unclear. Almost all of the literature involving artificial

DNA bases and isostere questions has been focused on derivatives of the purine dA, as opposed to dG.⁹⁻¹⁴ Literature searches for DNA polymerases plus dI or substituted dI (or dX) showed few examples, other than those already cited. Poly dI was a poor template for Moloney murine leukemia virus reverse transcriptase.³⁰ 2-Bromodeoxyinosine triphosphate was reported to be a substrate for *E. coli* pol I but few details were provided.³¹

We utilized the replicative DNA polymerase bacteriophage pol T7⁻ and translesion DNA polymerase IV from *Sulfolobus solfataricus* P2 (Dpo4) (A- and Y-family DNA polymerases, respectively) with an oligonucleotide containing dI, two halogen substituents (2-FdI and 2-BrdI), and a 2-oxo substituent (dX) (Figs. 1, 2). Pol T7⁻ is a kinetically well-behaved DNA polymerase for which crystal structures have been determined.³³⁻³⁵ The kinetics of its mechanism have been studied extensively,^{36,37} including bypass of DNA adducts.³⁸⁻⁴³ Dpo4⁴⁴ is capable of bypass of various DNA adducts and has been investigated in considerable detail in terms of both its function⁴⁵⁻⁴⁹ and structure.⁵⁰⁻⁵² In addition to these C2 modifications, we also changed the C6 oxo group of dG to sulfur (6-SdG; the nucleoside is a drug and has been studied previously regarding interactions with some DNA polymerases⁵³⁻⁵⁶). Both steady-state and pre-steady-state kinetic approaches were used. Both polymerases utilized template dI well, while 2-FdI, 2-BrdI, and dX significantly attenuated the catalytic efficiencies (k_{cat}/K_m) of both. The parameter k_{cat}/K_m ,⁵⁷ which should be considered a parameter itself and not a derivative of separate terms,⁵⁸ is related to the highest (rate-determining) activation free energy along the polymerization pathway ($\Delta G^\ddagger$) by transition state theory^{59,60}

$$\frac{k_{cat}}{K_m} = \frac{k_B T}{h} e^{-\frac{\Delta G^\ddagger}{RT}} \quad (\text{Eq. 1})$$

where k_B represents the Boltzmann constant, h is Planck's constant, R the universal gas constant, and T the thermodynamic temperature (see derivation of equations in supplemental data section). This rate-determining barrier may generally correspond to the substrate binding/release and the related conformational change or to the chemical (phosphodiester bond formation) step,⁵⁹ which we hypothesize would be significantly hindered by a combination of both steric and electrostatic clashes between the C2 substituent of the template and the O2 atom of the incoming dCTP as depicted in Fig. 3. This hindrance would be caused by a combination of enthalpic (steric and electronic repulsion) as well as entropic (non-optimal substrate orientation) factors. To address the validity of this hypothesis, a linear quantitative structure-activity relationship based on AMBER force field⁶¹ partial atomic charge and van der Waals radius of the C2 substituent was developed. Consistent with other work, Dpo4 was shown to be somewhat less sensitive to the substitution effect than pol T7⁻. The results allow discernment of some elements of nucleotide selectivity by DNA polymerases and insight into why the ubiquitous lesion dX miscodes.

Results

Primer Extension in the Presence of All Four dNTPs

Pol T7⁻ extended the primer (Table 1) to full-length (Fig. 4a) in the order dG (not shown) > dI > 2-FdI > 6-SdG > dX >> 2-BrdI. The 2-BrdI extension reaction showed mainly only 1-base incorporation. For Dpo4, the pattern was very similar (Fig. 4b).

One concern was that 2-FI could partially react with the amino group in Tris (buffer)^{62,63} although the incubation conditions (37 °C) were only for minutes, not hours. Analysis of the 2-FdI oligonucleotide after prolonged Tris incubation did not produce detectable amounts of a modified oligonucleotide (supplemental Fig. S2), and substituting MOPS buffer (which is not reactive because it contains a hindered tertiary amino group as opposed to the primary

amine in Tris) did not produce different results from those obtained using Tris in subsequent kinetic analyses (supplemental Fig. S3).

Analysis of Full-length Extension Products

In order to establish the identities of the bases inserted in the extension reactions, the extended primers were subjected to LC-MS analysis using a method previously developed in this laboratory.⁶⁴ Pol T7[−] showed only dCTP incorporation opposite dI, 2-FdI, and 6-SdG (Table 2). Dpo4 also inserted dCTP opposite dG and also opposite dI, 2-FdI, and 6-SdG (Table 2). Considerable incorporation of dATP was observed opposite dX with both polymerases, and small amounts of dTTP and dGTP incorporation were detected in the cases of pol T7[−] and Dpo4, respectively (Table 2, see also supplemental Figs. S4, S5 and Tables S1-S5). No frameshift products were found in any case. The limited full-length extension with the template containing 2-BrdI (Fig. 3) did not permit application of this technique in that case.

Steady-state Kinetics of Single-base Incorporation

With both pol T7[−] and Dpo4 (Tables 3, 4), the catalytic efficiencies (k_{cat}/K_m) for incorporation involving the normal Watson-Crick base pairs were similar. The higher efficiency for dCTP insertion opposite dG obtained with Dpo4 was reproducible; however, an efficiency similar to the other canonical base pairs ($\sim 0.03 \mu\text{M}^{-1} \text{s}^{-1}$) was observed in other sequence contexts (results not shown). Similar parameters were obtained with dI:dCTP or dC:dTTP pairing (Table 3), although the frequency of misinsertion was an order of magnitude higher than for dG:dCTP pairing. Substitution at the C2 position of dI was very disruptive to the function of both polymerases, with catalytic efficiency in the order 2-FdI > dX > 2-BrdI, although the relative decrease from dI to 2-FdI was less in the case of Dpo4 (Table 4) than pol T7[−] (Table 3). 6-SdG produced only a small attenuation in the catalytic efficiency of pol T7[−] ($\sim 75\%$ decrease) but a larger difference was seen with Dpo4 (Table 4), even considering the unusually high value measured for dG:dCTP pairing in this sequence context with this enzyme (see also supplemental Table S4). In the case of incorporation opposite dX, pol T7[−] preferentially inserted dCTP but also inserted the other three canonical dNTPs, at frequencies of 0.17-0.42 (Table 3). Dpo4 inserted all four dNTPs, with dTTP and dATP being most preferred.

The catalytic parameters indicated very limited misincorporation for dI, 2-FdI, and 6-SdG, consistent with full-length extension results reported in Table 2. dX and 2-BrdI produced significant miscoding with both polymerases. For both dX and 2-BrdI, the preferences for inserting dCTP vs. dTTP were reversed between pol T7[−] and Dpo4.

Steady-state parameters were collected for “next-base” extension past dG, dI, 2-FdI, and 6-SdG pairs with dCTP and dTTP (Table 1) with pol T7[−] and Dpo4 (supplemental Table S6). In all cases examined, extension beyond a dT paired opposite the modified base was highly unfavorable (cf. Table 2). These frequencies, along with the single-dNTP misinsertion results (Table 3), can explain the results observed in the LC-MS analyses of fully extended products. With pol T7[−] and dX, both insertion (opposite dX) and extension were very slow, and the preference for extension beyond an dX:dC pair explains the dominance of dC in the LC-MS results (Table 2). The pattern with Dpo4 and dX was less clear. Insertion opposite dX was slow for all dNTPs but somewhat more efficient with dTTP (Table 4). Extension past all dX:dN pairs was slow and the extension past an dX:dT was preferred (supplemental Table S6) but the LC-MS analysis showed no product with dTTP incorporation (Table 2). The apparent incongruity cannot be readily explained by back-folding/slippage,⁶⁴ in that the template was devoid of dA 5' of the dX, and we presume that dTTP can be inserted opposite dX and slowly extended by one base but extension stops for other reasons.

Pre-steady-state Kinetics of Single-base Incorporation

With pol T7⁻, sharp kinetic bursts were observed for all of the canonical incorporations (dG:dC and dA:dT pairs), the dI:dC pairs (Fig. 5b, 5e), and dCTP:6-SdG (Fig. 5j). A weaker burst was observed for the 2-FdI:dCTP pair (Fig. 5g) but none was seen with dX (Fig. 5h) or 2-BrdI (Fig. 5i). Dpo4 extension also showed (Fig. 6) burst kinetics for all of the normal incorporations and dI:dCTP (Fig. 6c) and dCTP:6-SdG (Fig. 6d). As with pol T7⁻, a weaker burst was seen with 2-FdI (Fig. 6f) but none with 2-BrdI or dX (Fig. 6g, 5h) (all with dCTP). In most cases the substitution of (*S*_p)-dCTPαS for dCTP (or (*S*_p)-dTTPαS for dTTP) did not appreciably change the kinetics except in the case of dCTP incorporation opposite 2-FdI (with both polymerases) (Figs. 5g, 6f), suggesting that the phosphodiester bond formation step is not rate-limiting in most cases.

The assays were repeated at varying dCTP concentrations (supplemental Figs. S5-S7) and the k_{pol} and $K_{\text{d,dCTP}}$ values were determined (Tables 5, 6). Misincorporation was also analyzed, but kinetic bursts were observed only in the cases of pol T7⁻ with dI:dTTP and dT:dITP pairing (supplemental Figs. S8, S9).

Spectroscopic Measurements

Each of the template (36-mer) oligonucleotides (Table 1) was paired with an 11-mer overlapping the modified site and used for CD spectroscopy and T_m measurements. The CD spectra of oligonucleotides containing G or any of the other modified bases examined (I, 2-FI, 2-BrI) were very similar (at 23 °C and pH 7.25) (supplemental Fig. S10).

The T_m value was lowered by substitution of dI for dG (Table 7, supplemental Fig. S11), and addition of a 2-fluoro, -oxo, or -bromo group lowered the T_m even more. The T_m value for 2-FdI was similar to that measured for *N*²,*N*²-Me₂G, a lesion that strongly disrupts polymerization by pol T7⁻, Dpo4, and human Y-family DNA polymerases.⁶⁵⁻⁶⁸

The pK_a values of the free nucleosides were determined using UV titration experiments (Table 8, supplemental Fig. S12). The pK_a values obtained for dG, dI, 2-FdI, and 2-BrdI were 9.2, 8.9, 4.7, and 6.0, respectively, and reflect the keto-enol equilibrium of the C6 carbonyl except in the case of dX (the C6 NMR chemical shifts verify the keto as opposed to the enol form, Fig. 1, *vide infra*). Therefore, 2-FdI exists in the enol form at neutral pH, at least in the free nucleoside. dX had a measured pK_a of 6.1 (Table 8), consistent with previous literature.⁶⁹⁻⁷³ In this case the equilibrium is more complex due to the ionization of the N3 proton as well as the N1 proton (Fig. 1b); previous work indicates that initial dissociation is for the N3 proton of dX rather than N1.^{69,71-75} Therefore, at physiological pH (pH of DNA polymerization buffer, 7.5) dX exists largely as a stable oxy anion (at C2) (Fig. 1b).^{28,70}

NMR Spectroscopy

The natural abundance ¹³C NMR spectra were assigned using literature precedents⁷⁶ and with the aid of HMBC data (Table 8). The ¹³C chemical shifts at the C6 atoms (all obtained at pH 7.5) are consistent with the pK_a values (Table 8) in that the 2-FdI and 2-BrdI values are ~167 ppm, apparently reflecting enol character, as opposed to the range of 159.6-161.7 ppm for dG, dI, and dX, which are in the keto (lactam) form. The ¹³C chemical shifts of the C2 atom varied considerably, from 145.0 to 160.3 ppm, but did not show any clear trend with regard to the electronegativity of the substituent atom. Neither chemical shift (C2 or C6) showed a correlation with the catalytic efficiency of either DNA polymerase (Tables 3, 4, 8). Attempts to measure natural abundance (N1) ¹⁵N NMR shifts were abandoned because of the low signal intensity.

Linear Activity Models

Experimental activation free energies ($\Delta G^\ddagger_{\text{exp}}$) of pol T7⁻ and Dpo4 were calculated based on Eq. 1 and collected in Table 9 with respect to the C2 substituent of the templating base. Merz-Kollman partial atomic charges of C2 substituents H, F, O, and Br calculated at the HF/6-31G(d) level of theory are also reported together with the corresponding van der Waals radii taken from the AMBER force field atom types HA, F, O2, and Br, respectively.⁶¹

A linear activity model was developed based on steric and electronic clash of the C2 substituent with the O2 atom of the incoming dCTP depicted in Fig. 3. The model $\Delta G^\ddagger_{\text{mod}} = (a \times r) + (b \times q) + n$, where $a = 9.86 \text{ kcal (mol } \epsilon)^{-1}$, $b = -3.37 \text{ kcal (mol } e_0)^{-1}$, and $n = -4.00 \text{ kcal mol}^{-1}$, provided the best agreement with the experiments for pol T7⁻ and was able to reproduce the experimentally observed order of substituent effects on the pol T7⁻ catalytic efficiency. As hypothesized, the positive coefficient a shows that the free energy barrier increases with the bulkiness of the substituent due to a steric clash, while the negative coefficient b indicates that the activation barrier increases with the negative charge of the substituent due to electrostatic repulsion by the adjacent cytosine oxygen. The abbreviated model based only on the steric contribution, $\Delta G^\ddagger_{\text{mod}} = (a \times r) + n$ (where $a = 9.23 \text{ kcal (mol } \epsilon)^{-1}$ and $n = -2.11 \text{ kcal mol}^{-1}$), was not able to reproduce the experimentally observed order of substituent effects on the pol T7⁻ catalytic efficiency. The truncated model based only on the electrostatic contribution, $\Delta G^\ddagger_{\text{mod}} = (b \times q) + n$ (where $b = -2.10 \text{ kcal (mol } e_0)^{-1}$ and $n = 13.8 \text{ kcal mol}^{-1}$), was also not able to reproduce the experimentally observed order of substituent effects on the pol T7⁻ catalytic efficiency.

The linear activity model $\Delta G^\ddagger_{\text{mod}} = (a \times r) + (b \times q) + n$, where $a = 6.07 \text{ kcal (mol } \epsilon)^{-1}$, $b = -3.95 \text{ kcal (mol } e_0)^{-1}$, and $n = 3.00 \text{ kcal mol}^{-1}$, provided the best agreement with the experiment for Dpo4 and was able to reproduce the experimentally observed order of substituent effects on Dpo4 catalytic efficiency. As in the case of pol T7⁻, the positive coefficient a shows that the free energy barrier increases with increasing steric demands of the substituent, while the negative coefficient b indicates that the same barrier increases with the negative charge of the substituent due to electrostatic repulsion by the adjacent cytosine oxygen. The abbreviated model $\Delta G^\ddagger_{\text{mod}} = (a \times r) + n$ (where $a = 5.34 \text{ kcal (mol } \epsilon)^{-1}$ and $n = 5.21 \text{ kcal mol}^{-1}$) was not able to reproduce the experimentally observed order of substituent effects on the Dpo4 catalytic efficiency. The truncated model $\Delta G^\ddagger_{\text{mod}} = (b \times q) + n$ (where $b = -3.17 \text{ kcal (mol } e_0)^{-1}$ and $n = 13.9 \text{ kcal mol}^{-1}$) was also not able to reproduce the experimentally observed order of substituent effects on the Dpo4 catalytic efficiency.

DISCUSSION

The focus of this investigation was to examine the effects of two naturally-occurring DNA bases, dI and dX, on two model DNA polymerases, bacteriophage pol T7⁻ and *S. solfataricus* Dpo4. dI is derived from deamination of dA and acts as a relatively high-fidelity mimic of G despite missing one hydrogen bond. Rates of dNTP insertion were not attenuated (relative to insertion of dCTP opposite dG or dTTP opposite dA).³ dX is a deamination product of G, known to be present at relatively high levels in mammalian cells,^{21,77} and strongly impeded polymerization and produced considerable misinsertion with both polymerases (Tables 3, 4). The molecular basis of the effect of dX was investigated further by substituting halogens (for oxygen) at the C2 position. These electronegative atoms had effects similar to the oxyanion of dX in that insertion of dCTP, but not dTTP, was attenuated. Little misincorporation was observed with 2-FdI with either DNA polymerase, as judged by either steady-state kinetics (Tables 3, 4) or LC-MS analysis of primer extension products (Table 2). None of the perturbations of the purine ring electron density (as manifested in the pK_a determinations and NMR chemical shifts, Table 8) explained the trends in enzymatic activity, nor did either the steric bulk at the C2 position or the substituent atom partial charge alone

(Table 9). The most satisfactory explanation for the effect was a combination of both the size and charge of the C2 substituent atom (Table 9); i.e., how much charge is “pushed” how far against the O2 atom of the pairing dCTP (Fig. 3). The same trend in the physicochemical parameters is also reflected in the T_m changes (Table 7), which indicates a similar role of steric-electronic clash between the C2 substituent of the template and the O2 atom of the C nucleotide in determining the thermal DNA duplex stability.

Halogens were selected as mimics of the anionic oxygen of dX because of their electronegativity, and the resulting attenuation of polymerase function (Tables 3, 4) has already been discussed. Previous studies indicated that the purine N3 proton of dX is largely ionized (i.e., the O2 enolate) at neutral pH ($pK_a \sim 6$) but the N1 proton is not⁶⁹⁻⁷³ (Fig. 1B), and our own results are consistent with this finding (Table 8). Interestingly, the low pK_a of 2-FdI (4.8) and the NMR spectra suggest that the N1 atom is in the ionized enol form (C6 oxyanion) at neutral pH (Table 8), yet activity opposite this base is better than with dX (Tables 3, 4 and Fig. 7a). 2-BrdI had a measured pK_a of 6.0 and the N1 proton should also be largely dissociated at neutral pH; however the selectivity for base insertion opposite 2-BrdI is similar to dX (Tables 3, 4). Overall, the pK_a values of the template bases and patterns of ionization are not well correlated to the order of catalytic efficiency (k_{cat}/K_m).

Linear activity models (Table 9) demonstrated that a combination of both van der Waals radii (steric clash) and Merz-Kollman partial atomic charges (electrostatic repulsion) was able to reproduce the experimentally observed order of substituent effects on the pol T7⁻ and Dpo4 catalytic efficiencies. These results implicate both steric and electrostatic clashes between the C2 substituent of the template base and the O2 atom of the incoming dCTP, depicted in Fig. 3, as the predominant factor controlling the insertion efficiency for the DNA polymerases examined here. The free energy barrier is increased by the negative charge of the C2 substituent in a similar fashion for both DNA polymerases as evidenced by nearly identical values of the linear coefficient b . This result can be rationalized by almost identical electrostatic repulsion between individual C2 substituent and the adjacent cytosine O2 atom obtained in both polymerases. On the other hand, pol T7⁻ is significantly more inhibited by the increasing van der Waals radius than Dpo4, as evidenced by a larger linear coefficient a . Because the van der Waals radius represents a direct measure of the bulkiness of the C2 substituent as well as of its distance from the C2 atom, the observed trend correlates well with the smaller enzyme active site of pol T7⁻.

A dI:dC pair has only two hydrogen bonds but the T_m was only slightly lower than for a dG:dC pair (Table 7), and the steady-state (Tables 3, 4) and pre-steady-state catalytic efficiencies (Tables 5, 6) were very similar to those seen with dG:dC and dA:dT pairs. The only disadvantage of a dI vs. dG template base was an order of magnitude decrease in fidelity for both enzymes (Tables 3, 4). In a similar vein, 6-SdG was rather similar to dG in its T_m (Table 7) and polymerization properties (Tables 3, 4, 5, 6). We conclude that the two Watson-Crick hydrogen bonds formed in dI:dC pairs (which are effective, at least with pol T7⁻, regardless of the “polarity” of which base is in the template and the dNTP) are sufficient for typical catalytic activity. The 2-amino group of dG does appear to provide some additional selectivity for dC in terms of the extra hydrogen bond (to the O2 atom of dC).

The steady-state and pre-steady-state kinetic analyses for the Dpo4 insertion reaction indicated that this polymerase was somewhat less sensitive to the C2 substituent of the template purine than pol T7⁻ (Tables 3, 4, and Fig. 7a). The differences caused by the fluorine and bromine substitutions are most dramatic (Fig. 7a, 7b). It is interesting to note that both polymerases still showed burst kinetics with 2-FI (but not dX or 2-BrdI) (Figs. 4, 5). The exact basis of the mechanism cannot be known in the absence of a crystal structure, but the larger size of the active site of Dpo4^{43,50} may accommodate an enolized 2-FdI paired with dCTP. One possibility

is that dX (and the halide analogs) disrupts the minor groove contacts between pol T7[−] and the nascent base pair; these contacts are not present in Dpo4 and thus the attenuation is lower.

Very recently Patro *et al.*⁷⁸ reported a study of purine C2 substituent atoms with human pol α , using steady-state kinetics. As in our own work with pol T7[−] and Dpo4, dI:dC pairing was similar to dG:dC pairing in terms of enzyme efficiency and the only disadvantage of dI was an enhanced miscoding frequency. dX was not examined in that study. Of interest, the addition of a C2 amino group to dATP or 6-chloropurine deoxyribonucleoside triphosphate increased the tendency to insert opposite a template dC (by pol α), possibly by increasing the number of hydrogen bonds from one to two. However, even in those cases where these two hydrogen bonds are possible, the catalytic efficiency was much less than in the case of dG:dC (and dI:dC), probably because the hydrogen bonding schemes were not Watson-Crick due to the lack of a proton on the N3 atom.⁷⁸ The results of another recent study⁷⁹ with Herpes Simplex Virus 1 DNA polymerase are also similar.

Our present findings have biological relevance in light of the relatively high levels of dX and dI found *in vivo*.²¹ These deaminated residues are not the only base damage products resulting from nitric oxide^{18,21,77} but the levels are on the same order of magnitude as the extensively studied 7,8-dihydro-8-oxo G and major malondialdehyde-dG adducts. The levels are considerably higher than those measured for the etheno adducts of dA and dG, although the latter showed a greater increase in level due to nitric oxide production.²¹ Several other potential DNA adducts were not found at levels >1 per 10⁷ bases *in vivo* in a mouse inflammation model²¹ and are assumed to have less contribution.

In summary, studies with these model replicative and translesion DNA polymerases use dI with high catalytic efficiency, as judged by both steady-state and pre-steady-state kinetic analyses. The only deficiency is a relatively small enhancement of the misincorporation frequency, but on the basis of dG (not dA). Thus, deamination of dA to form dI should yield an almost complete dA:dT to dG:dC transition mutation, as reported in one cellular experiment.²⁵ The conversion of dG to dX by deamination yields a variable degree of dG:dC to dA:dT transition mutations depending upon the particular DNA polymerase, in line with previous literature.^{15,19,28} Our studies with electronegative analogs show that the strong attenuation of binding and of catalytic activity (dCTP incorporation) can be explained by a combination of the steric and electrostatic clash of the charged oxygen atom (of dX, or a halogen in 2-FdI or 2-BrdI) with the O2 atom of dCTP (Fig. 3). The T_m differences are also consistent with this explanation (Table 7), and both a replicative and a translesion DNA polymerase show rather similar patterns (Fig. 7, Table 9). Accordingly, we expect that the mechanisms proposed here will apply to many DNA polymerases. Overall, these base structure-activity relationships provide a mechanistic understanding of the miscoding properties of two major DNA adducts derived from deamination.

Materials and Methods

Materials—Pol T7[−] and *E. coli* thioredoxin were expressed in *E. coli* and purified as described earlier.^{36,38,42} Dpo4 was expressed in *E. coli* and purified to electrophoretic homogeneity as described previously.⁶⁴ Unlabeled dNTPs were obtained from Amersham Biosciences (Piscataway, NJ), (S_p)-dCTP α S and (S_p)-dTTP α S were from Biolog Life Science Institute (Bremen, Germany), 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). The (deoxy)nucleoside dX was purchased from SigmaAldrich (St. Louis, MO), and (deoxynucleosides) dG and dI were purchased from Berry and Associates (Dexter, MI).

Synthesis of 2-Fdl—2-Fdl was synthesized as the protected nucleoside,⁸⁰ deprotected using DBU,⁸¹ and purified by HPLC using a Beckman ODS octadecylsilane (C₁₈) semi-preparative column (5 μ m, 10 mm $\times$ 250 mm). The following gradient program was used, with a flow rate of 1.5 ml min⁻¹: 0–15 min, linear gradient from 1 to 10% CH₃CN in H₂O; 15–20 min, linear gradient from 10 to 20% CH₃CN; 20–35 min, hold at 20% CH₃CN; 35–45 min, linear gradient from 20 to 100% CH₃CN; 45–65 min, hold at 100% CH₃CN; 65–70 min, linear gradient from 100 to 1% CH₃CN; 70–75 min, hold at 1% CH₃CN (all percentages v/v). The recovered product (84%) yield was characterized by its MS and NMR spectra. MS: *m/z* calcd for C₁₀H₁₁FN₄O₄ [M+H]⁺ 271.2, found 270.9; [M+Na]⁺ 293.2, found 293.1; calcd for purine ring fragment C₅H₃FN₄O 155.1, found 155.1. ¹H NMR (D₂O) δ 8.55 (s, 1H, N¹-H), 8.19 (s, 1H, C8-H), 6.37 (s, 1H, C-1'); ¹³C NMR (D₂O) δ 158.85 and 160.25 (C2), 149.65 (C4), 121.75 (C5), 168.24 (C6), 138.16 (C8). ¹⁹F NMR (D₂O) δ -52.95 (2-F).

Synthesis of 2-Brdl and Its Phosphoramidite

2-Bromo-O⁶-(2-(4-nitrophenyl)ethyl)-2'-dl—3',5'-O-[1,1,3,3-(Tetrakis-isopropyl)-1,3-disiloxanediyl]-6-O-[2-(4-nitrophenyl)ethyl]-2'-dG (170 mg, 0.26 mmol)⁸⁰ and SbBr₅ (140 mg, 0.39 mmol) were dried by co-evaporation of the solvent from solutions in anhydrous toluene (3 $\times$ 3 ml). Anhydrous CH₂Br₂ was added to the solution and cooled to -30 °C; *tert*-butylnitrite (108 μ l, 93 mg, 0.90 mmol) was then added slowly. The reaction was stirred at -30 °C for 2 h and then poured into a solution of NaHCO₃ and ice and extracted (3 $\times$) with CH₂Cl₂. The combined extracts were dried with Na₂SO₄, filtered, and concentrated *in vacuo*. The product was used for the next step without further purification.⁸¹

To a stirred solution of this fully protected 2-Brdl derivative (180 mg, 0.25 mmol) in tetrahydrofuran (5 ml) was added tetrabutylammonium fluoride (0.5 ml, 1.0 M in tetrahydrofuran). The reaction was stirred for 2 h at room temperature and the solvent was removed *in vacuo*. Purification on a Biotage SP1 medium pressure chromatography apparatus using an Si-25M silica gel column (1–12% CH₃OH gradient in CH₂Cl₂, v/v) afforded the desired product (92 mg, 77% yield): ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.58 (s, 1H, C8-H), 8.18 (d, 2H, *J* = 8.6 Hz, aromatic), 7.64 (d, 2H, *J* = 8.6 Hz, aromatic), 6.34 (t, 1H, *J* = 6.6 Hz, H-1'), 5.35 (d, 1H, *J* = 4.3 Hz, 3'-OH), 4.90 (t, 1H, *J* = 5.4 Hz, 5'-OH), 4.82 (t, 2H, *J* = 6.45 Hz, CH₂), 4.40 (m, 1H, H-3'), 3.86 (m, 1H, H-4'), 3.61–3.30 (m, 2H, H-5', H-5''), 3.30 (t, 2H, *J* = 6.45 Hz, CH₂), 2.66 (m, 1H, H-2'), 2.34 (m, 1H, H-2''). HRMS: calcd for C₁₈H₁₈N₅O₆BrNa [M + H]⁺, *m/z* 502.0338; found, *m/z* 502.0338 (+0.0 ppm).

2-Bromo-5'-O-(4,4'-dimethoxytrityl)-O⁶-[2-(4-nitrophenyl)ethyl]-2'-dl—2-Bromo-O⁶-(2-(4-nitrophenyl)ethyl)-2'-dl (*vide supra*, 92 mg, 0.19 mmol) was evaporated from anhydrous pyridine (3 $\times$ 3 ml) and then dissolved in anhydrous pyridine (5 ml). 4,4'-Dimethoxytrityl chloride (98 mg, 0.29 mmol) was added and the reaction was stirred for 5 h at room temperature under an Ar atmosphere. The solvent was removed *in vacuo*, and the residue was purified on a Biotage SP1 apparatus using an Si-25M silica gel column (0 to 6% CH₃OH gradient in CH₂Cl₂, with 1% (C₂H₅)₃N, v/v) to afford the desired product (110 mg, 74% yield): ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.49 (s, 1H, C8-H), 8.17 (d, 2H, *J* = 8.6 Hz, aromatic), 7.63 (d, 2H, *J* = 8.6 Hz, aromatic), 7.28 (m, 2H, aromatic), 7.18 (m, 7H, aromatic), 6.77 (d, 2H, *J* = 8.8 Hz, aromatic), 6.71 (d, 2H, *J* = 8.8 Hz, aromatic), 6.39 (t, 1H, *J* = 6.1 Hz, H-1'), 5.39 (d, 1H, *J*₁ = 4.7 Hz, 3'-OH), 4.80 (m, 2H, CH₂-CH₂), 4.45 (m, 1H, H-3'), 3.99 (m, 1H, H-4'), 3.70 and 3.68 (s, 6H, CH₃O), 3.30 (m, 2H, CH₂-CH₂), 3.22–3.26 (m, 1H, H-5'), 3.08–3.12 (m, 1H, H-5''), 2.80 (m, 1H, H-2'), 2.37 (m, 1H, H-2''). HRMS: calcd for C₃₉H₃₆N₅O₈BrNa [M + H]⁺, *m/z* 804.1645; found, *m/z* 804.1660 (+1.9 ppm).

2-Bromo-3'-O-[(N,N-diisopropylamino)-2-cyanoethoxyphosphinyl]-5'-O-(4,4'-dimethoxytrityl)-O⁶-(2-(4-nitrophenyl)ethyl)-2'-dl—2-Bromo-5'-O-(4,4'-

dimethoxytrityl)-*O*⁶-[2-(4-nitrophenyl)ethyl]-2'-dI (*vide supra*, 110 mg, 0.14 mmol) was evaporated from anhydrous pyridine (3 × 3 mL) and then dissolved in freshly distilled CH₂Cl₂ (3 mL). A solution of anhydrous 1*H*-tetrazole (460 μL of a 0.45 M solution in CH₃CN, 14 mg, 0.20 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphoramidite (67 μL, 64 mg, 0.21 mmol) were added and the reaction was stirred for 2 h at room temperature under an Ar atmosphere. The solvent was removed *in vacuo*, and the residue was purified on Biotage SP1 apparatus using an Si-25M silica gel column (10 to 90% gradient of ethyl acetate in hexane, plus 1% (C₂H₅)₃N, v/v) to afford the phosphoramidite reagent (75 mg, 54% yield): ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.50 (m, 1H, C8-H), 8.19 (m, 2H, aromatic), 7.64 (m, 2H, aromatic), 7.29 (m, 2H, aromatic), 7.16 (m, 7H, aromatic), 6.74 (m, 4H, aromatic), 6.43 (m, 1H, H-1'), 5.02 (m, 1H, H-3'), 4.78 (m, 2H, Ar-CH₂-CH₂), 4.28 (m, 1H, H-3'), 4.14 (m, 1H, POCH₂), 4.02 (m, 2H, H-4', POCH₂), 3.69 (s, 6H, CH₃O), 3.62 (m, 2H, POCH₂), 3.51 (m, 2H, CH(CH₃)₂), 3.30 (m, 4H, Ar-CH₂-CH₂, H-5', H-5''), 2.87 (m, 2H, CH₂CN), 2.66 (m, 2H, CH₂CN), 2.56 (m, 1H, H-2'), 2.27 (m, 1H, H-2''), 1.21 and 1.00 (m, 12H, CH(CH₃)₂). ³¹P NMR (DMSO-*d*₆, 121 MHz): δ 147.9, 147.2. HRMS: calcd for C₄₈H₅₄N₇O₉PBr [M + H]⁺, *m/z* 982.2904; found, *m/z* 982.2919 (+1.9 ppm).

2-BrdI—DBU (11 μL, 17 mg, 0.11 mmol) was added to a solution of 2-bromo-*O*⁶-(2-(4-nitrophenyl)ethyl)-2'-dI (52 mg, 0.11 mmol) in 95:5 CH₃CN/H₂O (v/v) (3 mL) and the reaction was heated at reflux for 24 h. The solvent was removed *in vacuo* and the residue was purified with a Biotage SP1 apparatus using a Si-25M silica gel column (5 to 30% CH₃OH gradient in CH₂Cl₂, v/v) to afford the product (23 mg, 64% yield): ¹H NMR (300 MHz, DMSO-*d*₆): δ 7.92 (s, 1H, C8-H), 6.16 (t, 1H, *J* = 6.1 Hz, H-1'), 5.27 (d, 1H, *J* = 3.6 Hz, 3'-OH), 5.14 (t, 1H, *J* = 5.3 Hz, 5'-OH), 4.35 (m, 1H, H-3'), 3.83 (m, 1H, H-4'), 3.60-3.48 (m, 2H, H-5', H-5''), 2.65-2.56 (m, 1H, H-2'), 2.21-2.14 (m, 1H, H-2''). ¹³C NMR (0.3 M potassium phosphate in H₂O/D₂O (95:5, v/v), pH 7.5): δ 144.26 (C2), 149.80 (C4), 123.24 (C5), 166.87 (C6), 138.39 (C8). HRMS: calcd for C₁₀H₁₁N₄O₄BrNa [M + H]⁺, *m/z* 352.9861; found, *m/z* 352.9865 (+1.1 ppm).

Oligonucleotide Synthesis—Unmodified 24-mer, 25-mers, and 36-mer (Table 1) were purchased from Midland Certified Reagent Co. (Midland, TX). Template 36-mers (HPLC-purified) containing dI, 2-FdI, dX, and 6-SdG were also purchased from Midland. The 36-mer oligonucleotide containing *N*²,*N*²-Me₂G was synthesized previously.⁶⁵ The template 36-mer with 2-BrdI was synthesized using modified phosphoramidite reagents^{62,84} with a Perseptive Biosystems Model 8909 DNA synthesizer, on a 1-μmol scale. Standard synthetic protocols using Glen Research reagents with *p*-(*tert*-butylphenoxy)acetyl-protected phosphoramidites were employed. The modified oligodeoxynucleotide was cleaved from the solid support and the exocyclic amino groups were deprotected in a single step using 0.1 M aqueous NaOH. DBU was used to remove the *O*⁶-(4-nitrophenethyl) group. A capillary gel electrophoretogram and mass spectrum of the 2-BrdI oligonucleotide are included in the Supplementary Data (supplemental Fig. S1).

Reaction and Analysis Conditions for Enzyme Assays—Standard DNA polymerase reactions were carried out in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 5 mM dithiothreitol, 100 μg bovine serum albumin mL⁻¹ (w/v), and 5% glycerol (v/v). Pol T7⁺ was used in reactions with 12.5 mM MgCl₂ at 25 °C and Dpo4 with 5 mM MgCl₂ at 37 °C.^{42,64,81,85} In the case of pol T7⁻, the enzyme was combined with 20-fold molar excess *E. coli* thioredoxin prior to incubations.^{36,38,81}

Primers were 5' end-labeled using T4 polynucleotide kinase/[γ-³²P]ATP and annealed to different templates by heating a 1:1 solution of oligonucleotide to 95 °C for 5 min and then slowly cooling to room temperature. All reactions were initiated by the addition of dNTP solutions containing MgCl₂ (at the indicated final concentration) to preincubated enzyme/DNA

mixtures. After reaction, 5- μ l aliquots of the reaction mixture were quenched with EDTA-formamide solution (50 μ l of 20 mM EDTA in 95% formamide (v/v) with 0.5% bromophenol blue (w/v) and 0.05% xylene cyanol (w/v)). Products were resolved using a 20% polyacrylamide (w/v) denaturing gel electrophoresis system containing 8 M urea, visualized using a Bio-Rad Molecular Imager FX instrument (Bio-Rad), and quantitated by phosphorimaging analysis using the Molecular Imager FX instrument with Quantity One software.

Primer Extension Assay with All Four dNTPs—Each of the DNA substrates (100 nM) was incubated with pol T7[−] or Dpo4 (0, 0.5, 2, 10, or 50 nM) in Tris-HCl buffer and reactions were initiated by adding dNTP·Mg²⁺ solution (100 μ M each dNTP and MgCl₂, conditions described above) (Fig. 3). At the indicated time points, the reactions were quenched with EDTA-formamide solution (*vide supra*) and analyzed by gel electrophoresis/phosphorimaging.

Steady-state Kinetic Analyses—A ³²P-labeled primer, annealed to either an unmodified or adducted template, was extended in the presence of varying concentrations of a single dNTP. The molar ratio of primer/template complex to pol T7[−] or Dpo4 was $\geq 10:1$. Polymerase concentrations and reaction times were adjusted to limit primer conversion to $< 20\%$.⁵ Reactions (20 μ l) were done with twelve different dNTP concentrations, quenched by the addition of EDTA-formamide solution (*vide supra*), and analyzed by gel electrophoresis, with subsequent quantitation of the results. Graphs of product formation versus dNTP concentration were fit using nonlinear regression (hyperbolic fits) in GraphPad Prism Version 3.0 (San Diego, CA) for the estimation of k_{cat} and K_m values.

Pre-steady-state Reactions—Rapid chemical quench experiments were performed using a model RQF-3 KinTek Quench Flow Apparatus (KinTek Corp., Austin, TX) with 50 mM Tris-HCl buffer (pH 7.4) in the drive syringes. Reactions were initiated by rapid mixing of ³²P-primer/template/pol T7[−] or Dpo4 mixtures (12.5 μ l) with the dNTP·Mg²⁺ complex (10.9 μ l) and then quenched with 0.6 M EDTA after varying incubation times. The reaction products (20 μ l) were mixed with 100 μ l of EDTA-formamide solution (*vide supra*), separated using denaturing gel electrophoresis, and quantitated. Pre-steady-state experiments with excess DNA or with excess pol T7[−] or Dpo4 were fit with equations 2 and 3, respectively, using nonlinear regression analysis in GraphPad Prism Version 3.0,

$$y = A(1 - \exp(-k_p t)) + k_{ss} t \quad (\text{Eq. 2})$$

$$y = A(1 - \exp(-k_p t)) \quad (\text{Eq. 3})$$

, where y is the concentration of product, A is the burst amplitude, k_p is the pre-steady-state rate of nucleotide incorporation, t is time, and k_{ss} is a steady-state velocity of nucleotide incorporation.^{36,86}

k_{pol} and $K_{d,dCTP}$ were estimated by performing pre-steady-state reactions at different dNTP concentrations and varying reaction times. Graphs of burst rates (k_{obs}) versus dNTP concentration were fit to the hyperbolic equation

$$k_{obs} = k_{pol} [\text{dNTP}] / ([\text{dNTP}] + K_{d,dCTP}) \quad (\text{Eq. 4})$$

where k_{pol} is the maximal rate of nucleotide incorporation and $K_{\text{d,dCTP}}$ is the equilibrium dissociation constant for dNTP.^{36,86}

Phosphorothioate Analysis—With the ^{32}P -primer annealed to a template, reactions were initiated by rapid mixing of ^{32}P -primer/template/polymerase mixtures (12.5 μl) with (S_{p})-dNTP $\alpha\text{S}\cdot\text{Mg}^{2+}$ complex (or dNTP $\cdot\text{Mg}^{2+}$) (10.9 μl) and then quenched with 0.6 M EDTA after varying reaction times. Products were analyzed as described for the pre-steady-state reactions (*vide supra*).

LC-MS/MS Analysis of Oligonucleotide Products from DNA Polymerase Reactions⁶⁴—Dpo4 (5 μM) or pol T7[−] (5 μM , plus 100 μM *E. coli* thioredoxin) was incubated with uracil-containing primer/template DNA (10 μM , see Table 2), all four dNTPs (1 mM each), and MgCl_2 (5 mM for Dpo4, 12.5 mM for pol T7[−]) in a final volume of 100 μl at 37 °C for Dpo4 or 25 °C for pol T7[−] for 4 h in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM dithiothreitol. Reactions were terminated by extraction of the remaining dNTPs using a size-exclusion chromatography column (Bio-Spin 6 chromatography column, Bio-Rad). Concentrated stocks of Tris-HCl, dithiothreitol, and EDTA were added to adjust the concentrations to 50 mM, 5 mM, and 1 mM, respectively. Next, *E. coli* uracil DNA glycosylase (20 units, Sigma-Aldrich) was added and the solution was incubated at 37 °C for 6 h to hydrolyze the uracil residues on the extended primer. The reaction mixture was then heated at 95 °C for 1 h in the presence of 0.25 M piperidine (to break the primer chain at the abasic sites), followed by removal of the solvent by centrifugation under vacuum. The dried sample was resuspended in 100 μl of H_2O for MS analysis.

LC-MS/MS analysis was performed on a Waters Acquity UPLC system (Waters, Milford, MA) connected to a Finnigan LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA), operating in the ESI negative ion mode. An Acquity UPLC BEH octadecylsilane (C_{18}) column (1.7 mm, 1.0 mm $\times$ 100 mm) was used with the following LC conditions: Buffer A contained 10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ plus 2% CH_3CN (v/v) and buffer B contained 10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ plus 95% CH_3CN (v/v). The following gradient program was used with a flow rate of 150 $\mu\text{l min}^{-1}$: 0–2.5 min, linear gradient from 100% A to 95% A/5% B (v/v); 2.5–6.0 min, linear gradient to 75% A/25% B (v/v); 6–6.5 min, linear gradient to 100% B; 6.5–8.0 min, hold at 100% B; 8.0–9.0 min, linear gradient to 100% A; 9.0–12.0 min, hold at 100% A. The temperature of the column was maintained at 50 °C. Samples were injected with an autosampler system. ESI conditions were as follow: source voltage 4 kV, source current 100 mA, auxiliary gas flow rate setting 20, sweep gas flow rate setting 5, sheath gas flow setting 34, capillary voltage -49 V, capillary temperature 350 °C, tube lens voltage -90 V. MS/MS conditions were: normalized collision energy 35%, activation Q 0.250, activation time 30 ms. The doubly (negatively) charged species were generally used for CID analysis. The calculations of the CID fragmentations of oligonucleotide sequences were done using a program linked to the Mass Spectrometry Group (Medicinal Chemistry) at the University of Utah (www.medlib.med.utah.edu/massspec).

pK_a Determinations—Aliquots (10 μl) of stock aqueous solutions containing the nucleosides dG, dI, 2-FdI, 2-BrdI, or dX (1.2 to 1.5 mM, based on ϵ_{254} 10.2 $\text{mM}^{-1}\text{cm}^{-1}$) were diluted with 400 μl of various 50 mM potassium phosphate buffers adjusted to the indicated pH values (2.0 to 12.0). UV spectra were recorded at 23 °C using a modified OLIS-Hewlett Packard 8452 diode array spectrophotometer (On-Line Instrument Systems, Bogart, GA) from 240 to 340 nm, with the corresponding buffer as reference. The absorbance difference $A_{254}-A_{320}$ was plotted vs pH. Data points were fit with Graphpad Prism (Graphpad, San Diego, CA) using nonlinear regression, fitting to the equation $Y = [(Y_{\text{min}} 10^{-X}) + (Y_{\text{max}} 10^{-\text{pK}_a})]/(10^{-X} + 10^{-\text{pK}_a})$,^{87,88} with SE calculated using the software program.

NMR Spectroscopy— ^{13}C NMR experiments were acquired using a 14.0 T Bruker magnet (Bruker, Billerica, MA) equipped with a Bruker AV-III console, operating at 600.13 MHz, and a 5-mm TCI cryogenically-cooled NMR probe. ^1H and ^{19}F NMR experiments were acquired using a 9.3 T Oxford magnet equipped with a Bruker AV console, operating at 400.13 MHz, and a 5-mm BBFO NMR probe. Samples were dissolved in either $\text{H}_2\text{O}/\text{D}_2\text{O}$ (95/5, v/v) or, for the ^{13}C work, 0.3 M potassium phosphate buffer (pH 7.5) in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (95:5, v/v). ^1H , ^{13}C , and ^1H - ^{15}N HSQC and ^1H - ^{13}C HMBC NMR spectra were acquired in 3.0-mm NMR tubes at 25 °C while spectra ^{19}F NMR spectra were acquired in standard 5 mm NMR tubes at 25 °C. Chemical shifts for ^{19}F and ^{13}C were referenced externally to hexafluorobenzene (−164.9 ppm) and TMS (0.0 ppm) respectively. For one-dimensional ^1H NMR, solvent suppression using the WATERGATE^{87,88} pulse sequence was necessary to reduce the intensity of the H_2O resonance. Experimental conditions for ^1H NMR spectra included 32 K data points, a 13 ppm sweep width, a recycle delay of 1.5 s, and 32–256 scans. For one-dimensional ^{19}F NMR spectra, experimental conditions included 65 K data points, 200 ppm sweep width, a recycle delay of 2.0 s, and 64 scans; for one-dimensional ^{13}C NMR, experimental conditions included 32 K data points, a 260 ppm sweep width, a recycle delay of 2.0 s, and 11 K scans. ^1H - ^{13}C HMBC experiments were acquired using a 2048×64 data matrix, a $J_{(\text{C-H})}$ value of 9 Hz for detection of long range couplings (resulting in an evolution delay of 55 ms), a $J_{1(\text{C-H})}$ filter delay of 145 Hz (34 ms) for the suppression of one-bond couplings, a recycle delay of 1.5 s, and 64 scans per increment. The HMBC data were also processed using a $\pi/2$ shifted squared sine window function and displayed in magnitude mode.

T_m Determinations—UV melting experiments were carried using a Varian Cary 4E spectrophotometer (Varian, Walnut Creek, CA). The 11-mer oligonucleotide 5'-CGCAGCGAGGA-3' and 36-mer 5'-TCGGCGTCCTCG*CTGCGTCTGCGGCTGGCTCGAGGC-3' form an 11-mer duplex containing G* in the middle of the duplex, where G* indicates dG, dI, 2-FdI, 2-BrdI, dX, 6-SdG, or N^2,N^2 -Me₂dG. The oligonucleotides (final duplex concentration ~1.7 μM) were dissolved in 1.0 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl and 1 mM EDTA in a 1.0 cm cuvette. The UV absorbance (A_{260} 0.7–1.0) was monitored at 1-min intervals with a 1 °C min^{−1} temperature gradient. The temperature was cycled between 15 and 80 °C. The error was estimated from the fit of the determined curve (increasing temperature data) to the sigmodal equation $Y = A_2 + (A_1 - A_2)/(1 + \exp[(X - T_m)/A_3])$, where A_1 , A_2 , and A_3 are three independent parameters. T_m values were matched with the estimates from the first derivatives (dA/dT) of the melting curves.

CD Analysis of Duplexes^{91–93}—CD spectra were recorded in 1-mm cuvettes with a JASCO J-810 instrument (JASCO, Easton, MD) in the Vanderbilt facility. A solution of the duplex of 11-mer/36-mer (6 μM) in 10 mM potassium phosphate buffer (pH 7.25) containing 100 mM NaCl was placed in the cell and spectra were recorded from 350 to 200 nm at 20 °C. The 11-mer oligonucleotide 5'-CGCAGCGAGGA-3' and 36-mer oligonucleotide 5'-TCGGCGTCCTCG*CTGCGTCTGCGGCTGGCTCGAGGC-3' were annealed to form a duplex (with G* indicating dG, dI, 2-FdI, or 2-BrdI). The absorbance in all CD studies was <0.20. CD output, in mdeg, was converted to standard mean residue ellipticity, $[\theta]$ (in mdeg $\mu\text{M}^{-1} \text{mm}^{-1}$), using the relationship

$$[\theta] = 100 \times \text{signal} / cl \quad (\text{Eq. 4})$$

where *signal* is the CD output in mdeg, *c* is the DNA concentration (in μM , based on total oligonucleotide duplex), and *l* is the cell path length (in mm).

Ab Initio Calculations—In order to reduce the computational costs, the 2'-deoxyribose moiety of dI, 2-FdI, 2-BrdI, and dX was replaced by a methyl group. The structures under consideration were subjected to full geometry optimization and subsequent vibrational analysis in the harmonic approximation at the Hartree-Fock (HF) level of theory using 6-31G(d) basis set encoded in the Gaussian 03 program.⁹⁴ The absence of imaginary vibrational frequencies indicated correctly performed minimizations.⁹⁵ Merz-Kollman partial atomic charges were finally calculated from the electrostatic potential surrounding the optimized structures.

Supplementary Material

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Abbreviations used

2-BrdI, 2-bromo-2'-deoxyinosine
 CID, collision-induced dissociation
 DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene
 dG, 2'-deoxyguanosine
 dI, 2'-deoxyinosine
 Dpo4, *S. solfataricus* P2 DNA polymerase IV
 ESI, electrospray ionization
 2-FdI, 2-fluoro-2'-deoxyinosine
 HMBC, heteronuclear multiple bond coherence
 HRMS, high resolution mass spectrometry
 LC, liquid chromatography
 MS, mass spectrometry
 MS/MS, tandem mass spectrometry
 MOPS, 4-morpholinepropanesulfonic acid
 N^2,N^2 -Me₂dG, N^2,N^2 -dimethyl-2'-deoxyguanosine
 pol, (DNA) polymerase
 pol T7⁻, bacteriophage polymerase T7/exonuclease⁻
 6-SdG, 6-thio-2'-deoxyguanosine
 dX, 2'-deoxyxanthosine.

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a **b** **Figure 1. Structures of purines**

a, Tautomers of 2-substituted purines. b, Tautomerization and ionization of dX.

Figure 2. Structures of dG, dA, dI, 2-FdI, 2-BrdI, dX, and N^2,N^2 -diMedG and their corresponding Connolly molecular surfaces in aqueous solution.³²

Figure 3.
Steric and electrostatic clash of the purine C2 atom substituent and deoxycytidine O2 atom.

Figure 4. Extension of ^{32}P -labeled primers

a, Extension with all four dNTPs present opposite dI, 2-FdI, 6-SdG, dX, and 2-BrdI by pol T7⁻. b, Extension opposite dI, 2-FdI, 6-SdG, dX, and 2-BrdI by Dpo4. Some of the lanes in Part B (dX and 2-BrdI) were run in a different set of experiments and are superimposed into the figure.

Figure 5. Pre-steady-state kinetics of nucleotide incorporation of dNTP or (Sp)-dNTPαS by pol T7

Pol T7⁻ (70 nM) was incubated with 120 nM 24-mer/36-mer primer:template complexes (³²P-labeled primer) in a rapid quench-flow instrument and mixed with 1 mM dNTP (■) (or (Sp)-dNTPαS (●)) plus 12.5 mM MgCl₂ to initiate reactions.

Figure 6. Pre-steady-state kinetics of incorporation of dNTP or (Sp)-dNTP α S by Dpo4
Dpo4 (70 nM) was incubated with 120 nM 24-mer/36-mer primer:template complexes (^{32}P -labeled primer) in a rapid quench-flow instrument and mixed with 1 mM dNTP plus 5 mM MgCl_2 to initiate reactions.

Figure 7. Comparison of effects of modifications at C2 on pol T7⁻ and Dpo4 kinetic parameters a, Steady-state, pol T7⁻ () and Dpo4 (); b, Dpo4 pre-steady-state.

Table 1

Oligodeoxynucleotides used in kinetic studies

24-mer	5'-GCCTCGAGCCAGCCGCAGACGCAG
25-mer	5'-GCCTCGAGCCAGCCGCAGACGCAG(T/C/G/A)
36-mer	3'-CGGAGCTCGGTCGGCGTCTGCGTC X CTCCTGCGGCT

~~X~~: dG, dA, dC, dT, dI, dA, 2-FdI, 2-BrdI, dX, 6-SdG, or N²,N²-Me₂dG.

Table 2

LC-MS/MS analysis of primer extension products

5' – GCCUCGAGUCAGCCGUAGACGUAG		3' – CGGAGCTCGGTCGGCGTCTGCGTCGCTCCTGCGGCT ^a		% of total product
pol T7 ^b	dI	AGCGAGGACGCCGA		9%
		AGCGAGGACGCCGAA		26%
		AGCGAGGACGCCGAC		19%
		AGCGAGGACGCCGAT		45%
	2-FdI	AGCGAGGACGCCGAA		25%
		AGCGAGGACGCCGAC		18%
		AGCGAGGACGCCGAG		11%
		AGCGAGGACGCCGAT		47%
	dX	AGCGAGGACGC		19%
		AGCGAGGACGCCG		4%
		AGCGAGGACGCCGA		21%
		AGCGAGGACGCCGAC		16%
		AGAGGAGGACGC		13%
		AGAGGAGGACGCCG		12%
		AGTGAGGACGCCGA		16%
	6-SdG	AGCGAGGACGCCGA		24%
		AGCGAGGACGCCGAA		20%
		AGCGAGGACGCCGAC		19%
		AGCGAGGACGCCGAT		37%
Dpo4 ^c	dG	AGCGAGGACGCCGA		17%
		AGCGAGGACGCCGAC		24%
		AGCGAGGACGCCGAA		59%
	dI	AGCGAGGACGCCGA		44%
		AGCGAGGACGCCGAC		17%
		AGCGAGGACGCCGAA		39%
	2-FdI	AGCGAGGACGCCGA		38%
		AGCGAGGACGCCGAC		20%
		AGCGAGGACGCCGAA		42%
	dX	AGCGAGGACGCCGA		24%
		AGCGAGGACGCCGAC		38%
		AGAGGAGGACGCCG		13%

^a **G**: dG, dI 2-FdI, 6-SdG, as indicated.

^c Reaction conditions: 5 μ M Dpo4, 10 μ M indicated 24/36-mer oligonucleotide complex, 1 mM each dNTP, 5 mM MgCl₂, 4 h at 37 °C. LC-MS: *m/z* 2.90-2.96 min; charge: -4; area: *m/z* value $\pm$ 0.2.

Table 3
Steady-state kinetic parameters for one-base incorporation by pol T7^a

Template base	dNTP	k_{cat} $s^{-1}, \times 10^{-3}$	K_m μM	k_{cat}/K_m $\mu M^{-1} s^{-1}$	Decrease (relative to dG:dCTP)	Misinsertion ratio ^b
dA	T	73 ± 3	0.17 ± 0.04	0.43	—	—
	C	18 ± 4	3160 ± 830	5.7 × 10 ⁻⁶	—	1.3 × 10 ⁻⁵
dT	A	620 ± 40	4.1 ± 1.0	0.15	—	—
	G	32 ± 3	2400 ± 340	1.3 × 10 ⁻⁵	—	8.7 × 10 ⁻⁵
	I	113 ± 5	890 ± 100	1.3 × 10 ⁻⁴	—	8.7 × 10 ⁻⁴
dC	G	230 ± 10	1.1 ± 0.4	0.21	1.9-fold	—
	I	150 ± 6	0.62 ± 0.10	0.24	—	1.1
	A	32 ± 2	3000 ± 380	1.1 × 10 ⁻⁵	—	5.1 × 10 ⁻⁵
dG	C	1200 ± 100	3.1 ± 0.6	0.39	—	—
	T	18 ± 1	800 ± 160	2.3 × 10 ⁻⁵	—	5.9 × 10 ⁻⁵
dI	C	150 ± 4	0.69 ± 0.10	0.22	1.8-fold	—
	T	52 ± 2	374 ± 26	1.4 × 10 ⁻⁴	—	6.4 × 10 ⁻⁴
2-FdI	C	44 ± 4	33 ± 6	1.3 × 10 ⁻³	300-fold	—
	T	20 ± 3	2040 ± 500	9.8 × 10 ⁻⁶	—	7.5 × 10 ⁻³
2-BrdI	C	3.3 ± 1.2	7100 ± 3300	4.7 × 10 ⁻⁷	830,000-fold	—
	T	3.0 ± 0.6	1800 ± 600	1.7 × 10 ⁻⁶	—	3.5
dX	C	13 ± 1	200 ± 20	6.5 × 10 ⁻⁵	6,000-fold	—
	G	21 ± 3	770 ± 250	2.7 × 10 ⁻⁵	—	0.42
	A	38 ± 9	3500 ± 1200	1.1 × 10 ⁻⁵	—	0.17
	T	24 ± 3	1800 ± 350	1.3 × 10 ⁻⁵	—	0.21
6-SdG	C	84 ± 2	1.5 ± 0.2	5.6 × 10 ⁻²	7-fold	—

Template base	dNTP	k_{cat} $s^{-1}, \times 10^{-3}$	K_m μM	k_{cat}/K_m $\mu M^{-1} s^{-1}$	Decrease (relative to dG:dCTP)	Misinsertion ratio ^b
	T	4.0 $\pm$ 0.3	680 $\pm$ 120	5.8 $\times 10^{-6}$	—	1.1 $\times 10^{-4}$

^a Conversion of primer to product was kept < 20% by adjusting the enzyme concentration and reaction time.

^b Misinsertion frequency = $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$.

Table 4
Steady-state kinetic parameters for one-base incorporation by Dpo4^a

Template base	dNTP	k_{cat} $s^{-1}, \times 10^{-3}$	K_m μM	k_{cat}/K_m $\mu M^{-1} s^{-1}$	Decrease (relative to dG:dCTP)	Misinsertion ratio ^b
dA	T	160 ± 10	6.1 ± 1.6	0.026	—	—
	A	22 ± 10	840 ± 230	2.6 × 10 ⁻⁵	—	1.0 × 10 ⁻³
	C	5.4 ± 0.8	1000 ± 300	5.4 × 10 ⁻⁶	—	2.1 × 10 ⁻⁴
	G	4.6 ± 0.3	770 ± 110	6.0 × 10 ⁻⁶	—	2.3 × 10 ⁻⁴
dT	A	360 ± 5	11 ± 0.8	0.033	—	—
	C	15 ± 1	940 ± 120	1.6 × 10 ⁻⁵	—	4.8 × 10 ⁻⁴
	G	42 ± 2	580 ± 60	7.2 × 10 ⁻⁵	—	2.2 × 10 ⁻³
	T	31 ± 1	750 ± 80	4.1 × 10 ⁻⁵	—	1.3 × 10 ⁻³
dG	C	930 ± 60	2.5 ± 0.8	0.37 ^c	—	—
	A	5.7 ± 0.2	490 ± 60	1.2 × 10 ⁻⁵	—	3.1 × 10 ⁻⁵
	G	6.3 ± 0.1	190 ± 20	3.3 × 10 ⁻⁵	—	9.0 × 10 ⁻⁵
	T	34 ± 2	780 ± 140	4.4 × 10 ⁻⁵	—	1.2 × 10 ⁻⁴
dC	G	540 ± 20	17 ± 2	0.032	—	—
	A	11 ± 2	1900 ± 540	5.8 × 10 ⁻⁶	—	1.8 × 10 ⁻⁴
	C	11 ± 2	1800 ± 430	6.1 × 10 ⁻⁶	—	1.9 × 10 ⁻⁴
	T	17 ± 2	1100 ± 200	1.5 × 10 ⁻⁵	—	4.7 × 10 ⁻⁴
dT	C	410 ± 20	17 ± 3	0.024	15-fold	—
	A	12 ± 1	200 ± 30	6.0 × 10 ⁻⁵	—	2.5 × 10 ⁻³
	G	1.6 ± 0.1	200 ± 30	8.0 × 10 ⁻⁶	—	3.3 × 10 ⁻⁴
	T	14 ± 3	820 ± 260	1.7 × 10 ⁻⁵	—	7.1 × 10 ⁻⁴
2-FdI	C	44 ± 2	6.8 ± 1.5	6.5 × 10 ⁻³	57-fold	—
	T	4.7 ± 0.4	200 ± 60	2.4 × 10 ⁻⁵	—	3.7 × 10 ⁻³

Template base	dNTP	k_{cat} $s^{-1}, \times 10^{-3}$	K_m μM	k_{cat}/K_m $\mu M^{-1} s^{-1}$	Decrease (relative to dG:dCTP)	Misinsertion ratio ^b
2-BrdI	C	19 ± 2	2000 ± 320	9.5 × 10 ⁻⁶	39,000-fold	—
	T	4.6 ± 0.8	990 ± 270	4.6 × 10 ⁻⁶	—	0.49
dX	C	11 ± 1	730 ± 200	1.5 × 10 ⁻⁵	25,000-fold	—
	G	10 ± 1	480 ± 140	2.1 × 10 ⁻⁵	—	1.4
	A	20 ± 1	720 ± 70	2.8 × 10 ⁻⁵	—	1.9
	T	35 ± 3	560 ± 120	6.3 × 10 ⁻⁵	—	4.2
6-SdG	C	160 ± 10	22 ± 4	0.0073	51-fold	—
	A	2.0 ± 0.2	490 ± 100	4.1 × 10 ⁻⁶	—	5.6 × 10 ⁻⁴
	G	1.3 ± 0.1	250 ± 40	5.2 × 10 ⁻⁶	—	7.1 × 10 ⁻⁴
	T	1.6 ± 0.1	390 ± 90	4.1 × 10 ⁻⁶	—	5.6 × 10 ⁻⁴

^aConversion of primer to product was kept < 20% by adjusting the enzyme concentration and the reaction time.

^bMisinsertion frequency = $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$.

^cSee footnote 3.

Table 5Pre-steady-state kinetic parameters for one-base incorporation by pol T7^{-a}

Template base	dNTP	$k_{\text{pol}}, \text{s}^{-1}$	$K_{\text{d,dCTP}}, \mu\text{M}$	$k_{\text{pol}}/K_{\text{d,dNTP}}, \mu\text{M}^{-1}\text{s}^{-1}$
dG	dCTP	28 ± 1.8	31 ± 6.6	0.90
dA	dTTP	260 ± 40	160 ± 40	1.6
dI	dCTP	230 ± 10	70 ± 9	3.3
	dTTP	0.25 ± 0.01	44 ± 7	5.7×10^{-3}
dC	dGTP	130 ± 8	44 ± 7	3.0
	dITP	210 ± 20	105 ± 20	2.0
dT	dATP	200 ± 20	84 ± 21	2.4
	dITP	0.57 ± 0.04	64 ± 13	8.9×10^{-3}
6-SdG	dCTP	45 ± 4	90 ± 20	0.5
2-FdI	dCTP	1.3 ± 0.1	130 ± 30	0.010

^aSee supplemental Fig. S17.

Table 6Pre-steady-state kinetic parameters for one-base incorporation by Dpo4^a

Template base	dNTP	k_{pol} s ⁻¹	$K_{d,dCTP}$ μM	$k_{pol}/K_{d,dNTP}$ μM ⁻¹ s ⁻¹
dA	dTTP	2.2 ± 0.2	58 ± 11	0.038
dT	dATP	3.0 ± 0.1	64 ± 7	0.047
dG	dCTP	3.0 ± 0.1	10 ± 1	0.30
dI	dCTP	2.8 ± 0.2	36c ± 6	0.078
2-FdI	dCTP	0.89 ± 0.04	16 ± 2	0.056
6-SdG	dCTP	2.4 ± 0.1	33 ± 5	0.073

^aSee supplemental Fig. S18.

Table 7 T_m values for 11-mer containing C hybridized to G or other residue

5'-CGCAGCGAGGA 3'-CGGAGCTCGGTCGGCGTCTGCGTC <u>X</u> CTCCTGCGGCT	
X	T_m (°C)
dG	49.5 ± 0.2
dI	45.4 ± 0.2
2-FdI	35.5 ± 0.3
2-BrdI	25.3 ± 1.1
dX	32.0 ± 0.4
6-SdG	44.7 ± 0.4
N^2,N^2 -Me ₂ dG	34.1 ± 0.2

Table 8p*K*_a values and NMR chemical shift data for purines

Purine	C2 substituent	p <i>K</i> _a	¹³ C Chemical shift ^a	
			C2 ppm	C6 ppm
dG	NH ₂	9.2 ± 0.1	154.8	159.9
dI	H	8.9 ± 0.0	147.0	159.6
2-FdI	F	4.7 ± 0.1	160.3	166.6
2-BrdI	Br	6.0 ± 0.1	145.0	167.4
dX	OH	6.1 ± 0.1	153.2	161.7

^aThe NMR chemical shifts were determined in 0.3 M potassium phosphate buffer (pH 7.5) in H₂O/D₂O (95:5, v/v) at 25 °C. The carbon chemical shifts are referenced externally to TMS (0.0 ppm).

Table 9

Linear quantitative structure-activity relationship based on partial atomic charge and van der Waals radius of the C2 substituent

Parameter	H	F	O	Br
$r, [\text{\AA}]^a$	1.46	1.75	1.66	2.22
$q, [e_0]^b$	0.094	-0.227	-0.776	-0.009
pol T7⁻				
$\Delta G_{\text{exp}}^\ddagger, \text{kcal mol}^{-1}{}^c$	10.3	13.4	15.2	18.1
$\Delta G_{\text{mod}}^\ddagger, \text{kcal mol}^{-1}$				
$(a \times r) + (b \times q) + n^d$	10.1	14.0	15.0	17.9
$(a \times r) + n^e$	11.4	14.0	13.2	18.4
$(b \times q) + n^f$	13.6	14.3	15.4	13.8
Dpo4				
$\Delta G_{\text{exp}}^\ddagger, \text{kcal mol}^{-1}{}^c$	12.1	12.9	16.7	17.0
$\Delta G_{\text{mod}}^\ddagger, \text{kcal mol}^{-1}$				
$(a \times r) + (b \times q) + n^g$	11.5	14.5	16.1	16.5
$(a \times r) + n^h$	13.0	14.6	14.1	17.1
$(b \times q) + n^i$	13.6	14.6	16.4	13.9

^a van der Waals radii.

^b Merz-Kollman partial atomic charges.

^c Experimental activation free energies.

^d Linear activity model $\Delta G_{\text{mod}}^\ddagger = (a \times r) + (b \times q) + n$, where $a = 9.86 \text{ kcal (mol \AA)}^{-1}$, $b = -3.37 \text{ kcal (mol } e_0)^{-1}$, and $n = -4.00 \text{ kcal mol}^{-1}$.

^e Linear activity model $\Delta G_{\text{mod}}^\ddagger = (a \times r) + n$, where $a = 9.23 \text{ kcal (mol \AA)}^{-1}$ and $n = -2.11 \text{ kcal mol}^{-1}$.

^f Linear activity model $\Delta G_{\text{mod}}^\ddagger = (b \times q) + n$, where $b = -2.10 \text{ kcal (mol } e_0)^{-1}$ and $n = 13.8 \text{ kcal mol}^{-1}$.

^g Linear activity model $\Delta G_{\text{mod}}^\ddagger = (a \times r) + (b \times q) + n$, where $a = 6.07 \text{ kcal (mol \AA)}^{-1}$, $b = -3.95 \text{ kcal (mol } e_0)^{-1}$, and $n = 3.00 \text{ kcal mol}^{-1}$.

^h Linear activity model $\Delta G_{\text{mod}}^\ddagger = (a \times r) + n$, where $a = 5.34 \text{ kcal (mol \AA)}^{-1}$ and $n = 5.21 \text{ kcal mol}^{-1}$.

ⁱ Linear activity model $\Delta G_{\text{mod}}^\ddagger = (b \times q) + n$, where $b = -3.17 \text{ kcal (mol } e_0)^{-1}$ and $n = 13.9 \text{ kcal mol}^{-1}$.