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B Family DNA Polymerases Asymmetrically Recognize Pyrimidines and Purines

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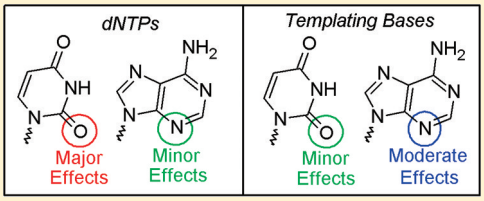
ABSTRACT: We utilized a series of pyrimidine analogues modified at O^2, N-3, and N^4/O^4 to determine if two B family DNA polymerases, human DNA polymerase α and herpes simplex virus I DNA polymerase, choose whether to polymerize pyrimidine dNTPs using the same mechanisms they use for purine dNTPs. Removing O^2 of a pyrimidine dNTP vastly decreased the level of incorporation by these enzymes and also compromised fidelity in the case of C analogues, while removing O^2 from the templating base had more modest effects. Removing the Watson−Crick hydrogen bonding groups of N-3 and N^4/O^4 greatly impaired polymerization, both of the resulting dNTP analogues and of natural dNTPs opposite these pyrimidine analogues when present in the template strand. Thus, the Watson−Crick hydrogen bonding groups of a pyrimidine clearly play an important role in enhancing correct dNTP polymerization but are not essential for preventing misincorporation. These studies also indicate that DNA polymerases recognize bases extremely asymmetrically, both in terms of whether they are a purine or pyrimidine and whether they are in the template or are the incoming dNTP. The mechanistic implications of these results with regard to how polymerases discriminate between right and wrong dNTPs are discussed.



Accurate replication of genomic DNA is critical for cellular survival and proliferation. Despite the complexity of the cellular DNA replication process, the overall error rates are remarkably low. Three discrete processes contribute to the accuracy of replication. First, replicative DNA polymerases typically exhibit relatively low error frequencies of $10^{-3}-10^{-6}$ errors per dNTP inserted.[1] Second, misincorporation events decrease the rate of elongation significantly, thereby allowing 3′−5′ exonucleolytic proofreading to occur and decreasing the error rate by 100-fold or so. Finally, postreplicative repair enzymes reduce the overall error frequency to roughly 10^{-9} errors per nucleotide inserted.[2]

The mechanisms by which various polymerases efficiently distinguish between correct and incorrect dNTP substrates during polymerization are not yet fully understood. Furthermore, different polymerases appear to utilize significantly different mechanisms.[3,4] Some low-fidelity enzymes, such as human primase, herpes primase, and Y family DNA polymerases, appear to largely utilize the Watson−Crick hydrogen bonding groups on the incoming (d)NTPs and the templating base to help identify correct and incorrect incorporation events.[5−10] On the other hand, various studies have shown that A and B family DNA polymerases do not require Watson−Crick hydrogen bond formation for dNTP incorporation.[11,12] B family polymerases use specific functional groups on the base of an incoming purine dNTP to prevent misincorporation and to enhance correct incorporation.[4,13−16] The methods used by A family polymerases are less well understood; while some studies indicate that shape selectivity may be critical for correct dNTP incorporation, others imply that shape is not important.[16−26] For example, Kool and co-workers showed that some A family polymerases efficiently incorporate 2,4-dihalotoluene dNTPs in a manner consistent with the enzyme using shape as a primary determinant.[27] However, these enzymes also readily incorporate purine dNTP opposite a templating T and dITP opposite a templating C,[19,28] even though the shapes of purine and hypoxanthine significantly vary from the shapes of adenine and guanine, respectively.

DNA polymerase α (pol α) is a key polymerase (along with primase, pol δ, and pol ε) required during nuclear DNA replication.[29] Pol α is a B family polymerase that typically exhibits low processivity, polymerizing ∼12 nucleotides before dissociating, and moderate fidelity, making $10^{-3}-10^{-5}$ errors per dNTP polymerized.[30,31] Biologically, pol α is responsible for the initial extension of primase-synthesized RNA primers in all new DNA strands. Pol α lacks 3′−5′ exonuclease activity; therefore, the incorporation of an incorrect dNTP results in the dissociation of pol α and subsequent association of a processive replicative polymerase with exonuclease activity (pol δ or pol ε).[29,32]

Pol α requires neither the formation of Watson−Crick hydrogen bonds nor a correctly shaped base pair for the rapid incorporation of a dNTP.[16,33,34] Instead, recent work has shown that during incorporation of dATP and related purine analogues, pol α employs a combination of positive and negative selectivity to ensure accuracy of replication. N-1 and N-3 serve as negative selectors and help prevent misincorporation, while N-1 and N^6 act as positive selectors and enhance correct incorporation.[4]

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Thus, a combination of positive and negative selectivity provides accuracy for pol α during dNTP incorporation, rather than shape or hydrogen bonding patterns. The HSV-1 polymerase, another B family DNA polymerase, uses the same general mechanisms as pol α, although the precise roles of N-1 and N^6 vary between the two enzymes.[14]

While the chemical features of purine bases have been examined with respect to their roles in correct incorporation and preventing misincorporation by pol α and herpes DNA polymerase, the contributions of the different functional groups on pyrimidines have not been examined. Accordingly, we examined the role of O^2, N-3, and N^4/O^4 of pyrimidine dNTPs and template bases for incorporation and fidelity with these two enzymes.

EXPERIMENTAL PROCEDURES

Materials. All reagents were of the highest quality commercially available. Unlabeled natural dNTPs were from Sigma, and radiolabeled dNTPs were from Perkin-Elmer. 2-Pyrimidinone dNTP (dZebTP) was from Trilink. Protected phosphoramidites were from Glen Research. Synthetic DNA oligonucleotides were purchased from IDT or Biosearch. The two-subunit p180−p70 polymerase complex was expressed in baculovirus-infected SF9 cells at the Tissue Culture Core Facility of the University of Colorado Health Sciences Center and purified as previously described,[35] with the exception that the enzyme was stored in 50% glycerol, 1 mM ethylenediaminetetraacetic acid, 50 mM Tris-HCl (pH 8.8), and 1 mM dithiothreitol. HSV-1 DNA polymerase (UL30−UL42 complex) was expressed and purified as previously described.[7]

5′-End Labeling of Primers and Annealing of Primer/Templates. DNA primers were 5′-^{32}P-labeled using polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. The labeled primer was gel purified and annealed to the template as previously described.[36,37]

Polymerization Assays. Assays were performed under steady-state conditions essentially as previously described[4,13,14] using either herpes simplex virus I DNA polymerase or pol α. Briefly, assays typically contained 1 μM 5′-[^{32}P]primer/template, 50 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 5% glycerol, and varying concentrations of natural or analogue dNTPs, in a total volume of 5−10 μL. Reactions were initiated via addition of enzyme, mixtures incubated at 37 °C for 5−30 min, and reactions quenched with an equal volume of gel loading buffer (formamide/0.05% xylene cyanol and bromophenol blue). Products were separated using denaturing gel electrophoresis (20% polyacrylamide and 8 M urea) and imaged using a Typhoon Phosphorimager (Molecular Dynamics). Kinetic parameters were determined by fitting data to the Michaelis−Menten equation using KaleidaGraph 4.0. All rates were normalized to the same final enzyme concentration (1 nM herpes pol and 5 nM pol α). The reported discrimination values were determined by comparing the efficiency of incorporation for the analogue (V_{max}/K_m) to the efficiency of incorporation for the corresponding natural nucleotide (V_{max}/K_m normalized to 1).

Synthesis of Nucleotide Analogues. C-2′-Deoxyribonucleosides (6AmPy, 6ClPy, 6MePy, and 6OPy) were synthesized as previously described.[38] 2OPy nucleoside and 5′-dimethoxytrityl 3′-phosphoramidites and oligonucleotides were synthesized as described using established procedures[39] on an Applied Biosystems 394 automatic DNA synthesizer.[40,41]

Synthesis of Nucleoside 5′-Triphosphates. Nucleosides were converted to nucleoside 5′-triphosphates as previously described.[39] Crude nucleoside triphosphates were purified by being loaded onto an anion-exchange column (Sephadex-DEAE A-25, Aldrich, pre-equilibrated in TEAB and then washed with H_2O) followed by elution with a 0 to 1 M TEAB gradient. Fractions were identified by MALDI mass spectrometry (negative M − 1 ion) with a THAP matrix. Collected fractions were evaporated and purified by reverse-phase (C18) high-performance liquid chromatography (HPLC) using a gradient of 0 to 50% MeCN in 20 mM triethylammonium acetate.

The purity was determined by the appearance of a single peak in HPLC (gradient from 0 to 60% MeCN in 20 mM TEAB in water) and the triphosphates identified by MALDI MS and ^{31}P NMR.

6AmPy dNTP: MS (MALDI, negative ion) 449 (M − 1) calcd 449; ^{31}P NMR (400 MHz, D_2O) δ −9.8 (bs, 2P, α-P, γ-P), −22.3 (m, 1P, β-P). 6ClPy dNTP: MS (MALDI, negative ion) 468 (M − 1) calcd 468; ^{31}P NMR (400 MHz, D_2O) δ −9.5 (bs, 2P, α-P, γ-P), −23.0 (m, 1P, β-P). 6MePy dNTP: MS (MALDI, negative ion) 448 (M − 1) calcd 448; ^{31}P NMR (400 MHz, D_2O) δ −10.4 (bs, 2P, α-P, γ-P), −22.9 (m, 1P, β-P). 6OPy dNTP: MS (MALDI, negative ion) 450 (M − 1) calcd 450; ^{31}P NMR (400 MHz, D_2O) δ −10.0 (bs, 2P, α-P, γ-P), −21.1 (m, 1P, β-P).

RESULTS

Previous studies have shown that B family DNA polymerases readily polymerize purine dNTP analogues whose base size, shape, and Watson−Crick hydrogen bonding pattern do not closely resemble those of canonical bases, while the canonical bases are only very rarely misincorporated.[4,16,33] The rate of incorporation of purine dNTPs opposite correct and incorrect template bases depends upon the effects of N-1, N-3, and N^6 via a combination of positive and negative selectivity. Herein, we probed the roles of O^2, N-3, and N^4/O^4 of pyrimidines to identify the similarities and differences between the interaction of B family polymerases with purines and pyrimidines (Figure 1).

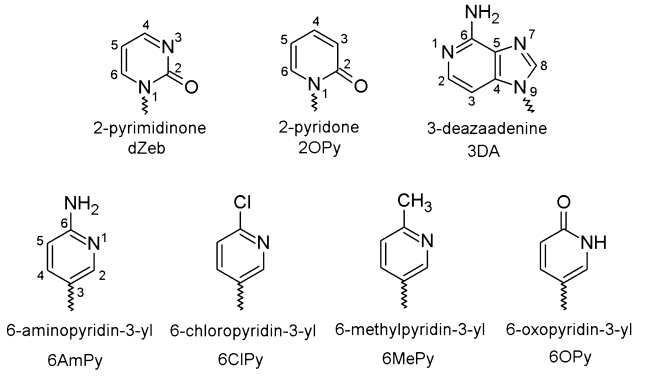

Figure 1. Structures, names, and abbreviations of analogue bases used.

The steady-state kinetic parameters for incorporation of both natural and analogue dNTPs were measured on synthetic primer/templates of defined sequence across from both natural and analogue templating bases. Discrimination values reflect the efficiency of correct dNTP incorporation compared to the efficiency of incorporation of the tested dNTP (V_{max}/K_m).

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Table 1. Natural Base Incorporations

dNTP	DNA-N	V_{max} (primer elongation min^{-1})	K_m (μM)	V_{max}/K_m (elongation μM^{-1} min^{-1})	discrimination[a]
			(a) Pol α		
dA	DNA-T	3.4 (0.1)	0.31 (0.05)	11	1
dT	DNA-A	3.2 (0.3)	1.7 (0.4)	1.8	1
dU	DNA-A	4.8 (0.4)	2.2 (0.4)	2.1	0.86
dC	DNA-G	9.6 (0.2)	1.2 (0.1)	8.4	1
dG	DNA-C	1.15 (0.09)	0.11 (0.3)	10	1
			(b) Herpes Pol		
dA	DNA-T	1.6 (0.05)	0.41 (0.06)	3.9	1
dT	DNA-A	1.7 (0.06)	1.3 (0.2)	1.3	1
dC	DNA-G	1.2 (0.02)	1.1 (0.1)	1.1	1
dG	DNA-C	6.5 (1.4)	2.6 (0.8)	2.5	1

[a]Discrimination values reflect the efficiency of dNTP incorporation compared to the efficiency of incorporation of the corresponding natural base pair. Values in parentheses are standard deviations.

Table 2. Effects of Watson–Crick Hydrogen Bonding Groups

dNTP	DNA-N	V_{max} (primer elongation min^{-1})	K_m (μM)	V_{max}/K_m (elongation μM^{-1} min^{-1})	discrimination[a]
			(a) Pol α		
dZeb	DNA-A	2.1 (0.3)	162 (61)	0.013	140
dZeb	DNA-G	4.6 (0.2)	8.5 (1.5)	0.54	16
dC	DNA-A	0.22 (0.01)	432 (65)	0.0005	3700
d2OPy	DNA-A	4.5 (0.5)	684 (242)	0.0041	450
d2OPy	DNA-T	2.6 (1.4)	335 (173)	0.0042	2600
d2OPy	DNA-C	nd	nd	nd	>10000
d2OPy	DNA-G	0.50 (0.3)	161 (17)	0.0015	5600
dA	DNA-2OPy	nd	nd	nd	>10000
dT	DNA-2OPy	nd	nd	nd	>10000
dC	DNA-2OPy	nd	nd	nd	>10000
dG	DNA-2OPy	nd	nd	nd	>10000
			(b) Herpes Pol		
d2OPy	DNA-A	0.48 (0.07)	430 (180)	0.0011	1200
d2OPy	DNA-T	0.29 (0.01)	120 (30)	0.0025	1900
d2OPy	DNA-C	nd	nd	nd	>20000
d2OPy	DNA-G	0.21 (0.02)	350 (80)	0.0006	1850

[a]Discrimination values reflect the efficiency of dNTP incorporation compared to the efficiency of incorporation of the corresponding natural base pair. nd indicates not detectable. Values in parentheses are standard deviations.

Table 1 shows the data for natural nucleotide incorporation by pol α and herpes pol. Both enzymes discriminated against incorrect natural dNTPs from 500- to >10000-fold.[14,16]

Role of Watson–Crick Hydrogen Bonding Groups. We initially examined the effect of removing N^4 from cytosine in the incoming dNTP using the base analogue dZebTP. Table 2a shows that pol α polymerized dZebTP 16-fold less efficiently opposite a templating G than it polymerized dCTP. Removing N^4 also had effects on fidelity because pol α discriminated against polymerizing dZebTP opposite a templating A by only 140-fold, somewhat less than the 3700-fold for misincorporation of dCTP. To examine the effects of removing the Watson–Crick hydrogen bonding groups from both N-3 and N^4/O^4, we utilized the base analogue 2-pyridone. Pol α strongly discriminated against polymerizing both 2-pyridone dNTP opposite the natural bases and the natural dNTPs opposite 2-pyridone. Thus, the Watson–Crick hydrogen bonding groups of a pyrimidine clearly play an important role in enhancing correct dNTP polymerization by pol α but are not essential for preventing misincorporation.

To determine if other B family DNA polymerases utilize the Watson–Crick hydrogen bonding groups of a pyrimidine like pol α, we examined HSV-1 DNA polymerase. Table 2b shows that HSV-1 pol also strongly discriminated against polymerizing 2-pyridone dNTP across from a natural template base. Because the amino acids that comprise the active sites of B family DNA polymerases are remarkably well conserved, these data suggest that this family of enzymes will generally discriminate against pyrimidine dNTP analogues lacking both Watson–Crick hydrogen bonding groups.

Role of the Minor Groove Hydrogen Bond Acceptor on a Pyrimidine dNTP. The importance of O^2, a hydrogen bond acceptor that lies in the minor groove (Figure 2), for generation of base pairs involving pyrimidines was examined using a series of analogues that lacked O^2. So that N-3 and N^4/O^4 (if present) maintained the hybridization states appropriate for either C or T and to avoid adding a positive charge to the ring, N-1 was also converted to carbon such that the nucleosides were C-glycosides. Three of the bases, 6MePy, 6ClPy, and 6AmPy, are C analogues that can form one, one, and two Watson–Crick hydrogen bonds with G, respectively, while 6OPy is a T analogue that can form two Watson–Crick hydrogen bonds with A (Figure 1). As a T analogue, 6OPy also lacks the methyl group found at C-5 of T. To first test the possibility that the absence of this methyl group was significant, we compared polymerization of dUTP and dTTP (Table 1).

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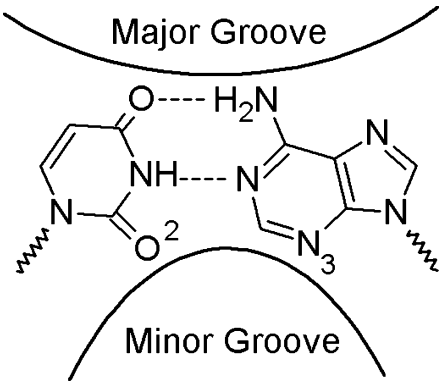

Figure 2. Major and minor grooves in DNA.

The similar kinetic parameters for dUTP and dTTP polymerization indicate that the absence of the methyl has little if any effect on polymerization.

Pol α strongly discriminated against polymerizing 6OPy dNTP opposite a templating A [120-fold discrimination (Table 3a)] even though this base can form two Watson–Crick hydrogen bonds. On the other hand, the loss of O^2 did not result in major changes in the rate of misincorporation opposite a template T, C, or G compared to that of the natural dTTP, indicating that O^2 is not a major determinant in preventing misincorporation of dTTP. HSV-1 DNA polymerase likewise discriminated against polymerization of 6OPy dNTP, with the only difference between HSV-1 DNA polymerase and pol α being the larger effect of losing O^2 with the HSV-1 enzyme [610-fold opposite a template A (Table 3b)].

Removing O^2 from dCTP had larger and more diverse effects than the corresponding modification of dUTP. We synthesized and tested three analogues, 6ClPy dNTP, 6MePy dNTP, and 6AmPy dNTP, capable of forming one, one, and two Watson–Crick hydrogen bonds with G, respectively (Table 4). Pol α did not detectably polymerize 6AmPy dNTP opposite G, even though two Watson–Crick hydrogen bonds could be formed. Likewise, it did not detectably polymerize 6ClPy dNTP or 6MePy dNTP opposite a templating G. Second, pol α misincorporated d6APy dNTP at higher rates than it did dCTP, suggesting that O^2 of dCTP analogues plays a greater role in fidelity than O^2 of dTTP does. HSV-1 DNA polymerase strongly discriminated against 6AmPy, 6ClPy, and 6MePy dNTPs opposite any template base, more so than pol α did and analogous to this enzyme's stronger discrimination against 6OPy dNTP polymerization.

Role of the Minor Groove Hydrogen Bond Acceptor on the Templating Base. We extended these studies to determine how removing O^2 from a templating pyrimidine would affect polymerization of a natural dNTP. The loss of O^2 from a templating T significantly reduced the level of polymerization of dATP even though two Watson–Crick hydrogen bonds can still form (Table 5). Polymerization of noncognate dNTPs was not affected, indicating that O^2 of the template T does not play an important role in fidelity. These data also indicate that removing O^2 from T in either the incoming dNTP or the templating base has very similar effects.

Removing O^2 from a templating C had relatively small effects on the efficiency of dGTP polymerization [14-fold (Table 5)], in contrast to the huge effect observed upon removal of O^2 from the incoming dCTP [undetectable polymerization opposite G (Table 4a)]. Misincorporation opposite a templating 6AmPy (Table 5) was not significantly different than misincorporation opposite a templating C, a result that stands in contrast to the decreased fidelity of 6AmPy dNTP polymerization. Thus, the effects of removing O^2 from C are highly asymmetric depending upon whether one looks at the incoming dCTP or templating C, and quite different from the effects of removing O^2 from T.

The role of the minor groove hydrogen bond acceptor in a purine, N-3, was likewise examined by measuring incorporation of dNTPs opposite 3-deazaadenine (3DA). Table 6 shows that this loss of N-3 impaired incorporation of the cognate dTTP but did not greatly impact incorporation of noncognate dNTPs. Thus, purine N-3 plays decidedly different roles depending upon whether the purine nucleotide is the dNTP or the templating nucleotide. In the dNTP, N-3 has minimal impact on correct dNTP incorporation but plays a major role in preventing misincorporation,[4] while in the template, N-3 is important for correct incorporation but has a minimal effect in preventing misincorporation (Table 6).

DISCUSSION

The effects of removing heteroatoms from pyrimidine bases in both the template and incoming dNTP on two B family DNA polymerases were examined. Unlike the very small effects of removing N-3 from a purine dNTP,[4] removing the functionally equivalent O^2 of a pyrimidine dNTP greatly decreased the rate of incorporation by B family DNA polymerases and also

Table 3. Effects of O^2 in dTTP

dNTP	DNA-N	V_{max} (primer elongation min^{-1})	K_m (μM)	V_{max}/K_m (elongation μM^{-1} min^{-1})	discrimination[a]
			(a) Pol α		
d6OPy	DNA-A	2.27 (0.07)	146 (13)	0.016	120
d6OPy	DNA-T	nd	nd	nd	>10000
d6OPy	DNA-C	L	L	0.018	600
d6OPy	DNA-G	nd	nd	nd	>10000
			(b) Herpes Pol		
d6OPy	DNA-A	0.42 (0.12)	200 (110)	0.0022	610
d6OPy	DNA-T	nd	nd	nd	>20000
d6OPy	DNA-C	nd	nd	nd	>20000
d6OPy	DNA-G	nd	nd	nd	>20000

[a]Discrimination values reflect the efficiency of dNTP incorporation compared to the efficiency of incorporation of the corresponding natural base pair. nd indicates not detectable. L indicates that the rate increased linearly with an increasing dNTP concentration, thus making it impossible to calculate K_m or V_{max}. V_{max}/K_m was calculated from the slope of the line. Values in parentheses are standard deviations.

Table 4. Effects of O^2 in dCTP

dNTP	DNA-N	V_{max} (primer elongation min^{-1})	K_m (μM)	V_{max}/K_m (elongation μM^{-1} min^{-1})	discrimination[a]
			(a) Pol α		
d6AmPy	DNA-A	0.83 (0.03)	99 (9)	0.0084	220
d6AmPy	DNA-T	L	L	0.037	270
d6AmPy	DNA-C	L	L	0.0096	1100
d6AmPy	DNA-G	nd	nd	nd	>10000
d6MPy	DNA-A	0.31 (0.05)	675 (210)	0.0005	4000
d6MPy	DNA-T	nd	nd	nd	>10000
d6MPy	DNA-C	nd	nd	nd	>10000
d6MPy	DNA-G	nd	nd	nd	>10000
d6ClPy	DNA-A	1.03 (0.03)	43 (3)	0.024	76
d6ClPy	DNA-T	nd	nd	nd	>10000
d6ClPy	DNA-C	nd	nd	nd	>10000
d6ClPy	DNA-G	nd	nd	nd	>10000
dC	DNA-A	0.22 (0.01)	432 (65)	0.0005	3700
dC	DNA-T	0.18 (0.01)	67 (8)	0.0027	4100
dC	DNA-C	nd	nd	nd	>10000
			(b) Herpes Pol		
6AmPy	DNA-A	0.075 (0.007)	120 (40)	0.00063	2100
6AmPy	DNA-T	nd	nd	nd	>20000
6AmPy	DNA-C	nd	nd	nd	>20000
6AmPy	DNA-G	0.043 (0.04)	110 (40)	0.0004	2800
6ClPy	DNA-A	0.71 (0.04)	1900 (200)	0.00037	3700
6ClPy	DNA-T	nd	nd	nd	>20000
6ClPy	DNA-C	nd	nd	nd	>20000
6ClPy	DNA-G	nd	nd	nd	>20000
6MePy	DNA-A	L	L	0.00011	12000
6MePy	DNA-T	nd	nd	nd	>20000
6MePy	DNA-C	nd	nd	nd	>20000
6MePy	DNA-G	nd	nd	nd	>20000

[a]Discrimination values reflect the efficiency of dNTP incorporation compared to the efficiency of incorporation of the corresponding natural base pair. nd indicates not detectable. L indicates that the rate increased linearly with an increasing dNTP concentration, thus making it impossible to calculate K_m or V_{max}. V_{max}/K_m was calculated from the slope of the line. Values in parentheses are standard deviations.

Table 5. Effects of O^2 in the Template

dNTP	DNA-N	V_{max} (primer elongation min^{-1})	K_m (μM)	V_{max}/K_m (elongation μM^{-1} min^{-1})	discrimination[a]
			Pol α		
dA	DNA-6OPy	0.38 (0.03)	4 (1)	0.093	120
dT	DNA-6OPy	0.11 (0.01)	66 (16)	0.0016	6900
dG	DNA-6OPy	0.10 (0.01)	71 (16)	0.0014	7700
dC	DNA-6OPy	nd	nd	nd	>10000
dA	DNA-6AmPy	0.8 (0.1)	642 (184)	0.0012	8100
dT	DNA-6AmPy	0.12 (0.01)	73 (20)	0.0016	6400
dC	DNA-6AmPy	nd	nd	nd	>10000
dG	DNA-6AmPy	0.12 (0.01)	0.16 (0.33)	0.74	14

[a]Discrimination values reflect the efficiency of dNTP incorporation compared to the efficiency of incorporation of the corresponding natural base pair. nd indicates not detectable. Values in parentheses are standard deviations.

Table 6. Effects of Removing N-3 from a Templating dA

dNTP	DNA-N	V_{max} (primer elongation min^{-1})	K_m (μM)	V_{max}/K_m (elongation μM^{-1} min^{-1})	discrimination[a]
			Pol α		
dA	DNA-3DA	0.13 (0.02)	134 (66)	0.0010	1900
dT	DNA-3DA	33 (2)	178 (30)	0.037	50
dC	DNA-3DA	nd	nd	nd	>10000
dG	DNA-3DA	nd	nd	nd	>10000

[a]Discrimination values reflect the efficiency of dNTP incorporation compared to the efficiency of incorporation of the corresponding natural base pair. nd indicates not detectable. Values in parentheses are standard deviations.

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compromised fidelity in the case of C analogues. Removing O^2 from the templating base had more modest effects. Finally, removing the Watson−Crick hydrogen bonding groups from pyrimidines greatly impaired polymerization of the resulting dNTPs, as well as polymerization of natural dNTPs opposite these bases.

Removing O^2 from pyrimidine dNTPs had surprisingly large effects on incorporation by both HSV-1 polymerase and pol α (Tables 2 and 3). Even with analogues that could still form two Watson−Crick hydrogen bonds, correct incorporation was significantly impaired. The effects on fidelity varied from no effect to moderate increases in the rate of misincorporation (as defined by the polymerization of the analogue dNTP opposite a templating base with which it cannot form any normal Watson−Crick hydrogen bonds).

These very large effects of removing O^2 from an incoming pyrimidine dNTP contrast with the effects of removing N-3 from a purine dNTP.[4] Both groups reside within the minor groove in similar locations and are hydrogen bond acceptors (Figure 2), yet whereas converting N^3 of dATP or dGTP to a carbon results in only small effects on correct polymerization and either significantly increases or has no effect on polymerization of an incorrect purine dNTP, removing O^2 of a pyrimidine dNTP cripples polymerization generally.

While not as dramatic as the differences observed for polymerization of purine dNTPs versus pyrimidine dNTPs, comparing the effects of removing O^2 from dUTP and dCTP revealed significantly different effects. Removing O^2 from dCTP more severely impacted correct incorporation and, in the case of pol α, had a greater effect on fidelity. These differences provide further support for the idea that the mechanisms by which DNA polymerases recognize each of the canonical base pairs are nonidentical. Previous studies showed that the identical modification to different bases can have vastly different effects.[28,42] Additional support for this idea comes from biophysical studies by Waksman and co-workers, who found that ternary E−DNA−dNTP structures of KlenTaq varied accordingly with the identity of the dNTP-templating base examined.[43] Furthermore, the enzyme showed different dynamics during replication of each canonical base pair.[44] Thus, it is probably inappropriate to consider a DNA polymerase as a "simple" machine that simply pairs an incoming dNTP with the templating base. Rather, the polymerase may actively read the templating base and then alter the chemistry of base pair recognition depending upon the templating base.

The effects of removing O^2 from a templating U were very similar to the effects of removing O^2 from the incoming dUTP during pol α-catalyzed DNA synthesis. In contrast, whereas removing O^2 from an incoming dCTP severely impacted both correct incorporation and fidelity, eliminating O^2 from a templating C had but modest effects on polymerization of a correct dGTP and no significant effects on misincorporation of noncognate dNTPs. In the case of adenine, removing N-3 from a templating A had a greater impact on polymerization efficiency [50-fold decrease (Table 6)] than removing N-3 from the incoming dATP (5-fold decrease[4]) during generation of an A-T base pair. These differences provide further evidence that DNA polymerases can recognize nucleotides very asymmetrically depending upon whether they are in the template or are the incoming dNTP, and that minor groove chemistry plays a major role in this asymmetry.

Potentially, three alternative mechanisms could explain these very different results for the effects of removing purine N-3 and pyrimidine O^2. The different steric effects of altering N-3 and O^2 could explain the differences. Whereas replacing N-3 of a purine with C has an only small steric effect, removing O^2 of a pyrimidine has a large effect. Alternatively, the polymerases may differentially interact with the minor groove side of incoming purine and pyrimidine dNTPs, thereby causing the vastly different effects. The active sites of the B family polymerases are remarkably well conserved, especially the group of amino acids that surround the incipient base pair between the incoming dNTP and the templating base. The B family polymerase from RB69 has been extensively studied structurally and provides potential clues for these differences.[45−47] While there are no obvious hydrogen bonding groups in RB69 polymerase that could interact with O^2 or N-3, the electron deficient edge of a tyrosine sits squarely in the minor groove of the incipient base pair (Y567 in RB69 polymerase, Y957 in pol α, and Y818 in HSV-1 DNA polymerase[4,45,47]). In the ternary RB69 polymerase−DNA−dTTP complex, this tyrosine interacts with O^2 of the incoming dTTP.[45] It is unknown if this tyrosine adopts a similar conformation during polymerization of other dNTPs or if it adopts different conformations at different points during the catalytic cycle. Importantly, differential interaction of this tyrosine with a purine N-3 versus a pyrimidine O^2 would explain the differences between removing the purine N-3 and pyrimidine O^2. Likewise, the different effects of removing O^2 from an incoming dCTP versus a templating C could be mediated by this tyrosine; for example, it could adopt different positions depending upon the identity of the templating base. This would significantly alter its interactions with the electron rich N-3 of a purine or O^2 of a pyrimidine and, depending upon the functional role of these interactions, alter correct and/or incorrect dNTP polymerization. Finally, these differential results could reflect the electronic changes in the aromatic rings caused by removal of purine N-3 and pyrimidine O^2. For example, removing N-3 will decrease the π electron density of the ring. The altered electron density could impact both stacking onto the neighboring bases and interactions with two conserved amino acids that interact with the π faces of the templating and dNTP bases (asparagines and serine), thereby affecting polymerization. Importantly, the remarkably well-conserved chemistry of this region of the active site among different B family polymerases[48] suggests that the results observed for HSV-1 polymerase and pol α will be similar to those for other members of this family.

The Watson−Crick hydrogen bonding groups of a pyrimidine clearly play an important role in enhancing correct dNTP polymerization but are not essential for preventing misincorporation. In the absence of any Watson−Crick hydrogen bonding groups (i.e., 2-pyridone), neither pol α nor HSV-1 polymerase efficiently polymerized a canonical dNTP. Adding N-3 back to the pyridine significantly increased the rate of polymerization of the resulting dZebTP opposite a templating G, demonstrating the key role of this group in promoting efficient polymerization. As noted earlier, however, the ability to form two Watson−Crick hydrogen bonds did not by itself ensure efficient polymerization. Additionally, the efficient polymerization of dZebTP provides further evidence that a correctly shaped base pair is not needed for efficient incorporation of dNTP by pol α.

These results have significant implications for the design of novel base pairs that are incorporated efficiently and accurately by polymerases, particularly if one would like to design bases that are "purine-like" and "pyrimidine-like". Efficient and accurate replication of any novel base pair requires that the chemical

features of the two bases satisfy the requirements of the polymerase. However, the data presented herein show that polymerases can exhibit distinctly different requirements for purine and pyrimidine dNTPs (e.g., the minor groove hydrogen bond acceptors N-3 and O^2 on purines and pyrimidines). Furthermore, the chemical requirements can vary depending upon whether the base is on the incoming dNTP or on the template strand. For example, while accurate and efficient incorporation of dCTP absolutely requires O^2 of dCTP, this is not true for a templating C. These large differences in chemical requirements for even the different natural bases imply that different unnatural bases will also have diverse and potentially unpredictable requirements, thereby greatly complicating efforts to rationally design novel base pairs.

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ABBREVIATIONS

6AmPy, 6-aminopyridin-3-yl; 6ClPy, 6-chloropyridin-3-yl; 3DA, 3-deazaadenine; MALDI, matrix-assisted laser desorption ionization; 6MePy, 6-methylpyridin-3-yl; 2OPy, 2-pyridone; 6OPy, 6-oxopyridin-3-yl; pol α, DNA polymerase α; pol δ, DNA polymerase δ; pol ε, DNA polymerase ε; SD, standard deviation; TEAB, triethylammonium bicarbonate; THAP, 2′,4′,6′-trihydroxyacetophenone; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloric acid; dZeb, 2-pyrimidinone (zebularine).

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