Formation of Purine−Purine Mispairs by *Sulfolobus solfataricus* DNA Polymerase IV[†]

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ABSTRACT: DNA damage that stalls replicative polymerases can be bypassed with the Y-family polymerases. These polymerases have more open active sites that can accommodate modified nucleotides. The lack of protein−DNA interactions that select for Watson−Crick base pairs correlate with the lowered fidelity of replication. Interstrand hydrogen bonds appear to play a larger role in dNTP selectivity. The mechanism by which purine–purine mispairs are formed and extended was examined with *Solfolobus solfataricus* DNA polymerase IV, a member of the RAD30A subfamily of the Y-family polymerases, as is pol η. The structures of the purine−purine mispairs were examined by comparing the kinetics of mispair formation with adenine versus 1-deaza- and 7-deazaadenine and guanine versus 7-deazaguanine at four positions in the DNA, the incoming dNTP, the template base, and both positions of the terminal base pair. The time course of insertion of a single dNTP was examined with a polymerase concentration of 50 nM and a DNA concentration of 25 nM with various concentrations of dNTP. The time courses were fitted to a first-order equation, and the first-order rate constants were plotted against the dNTP concentration to produce k_{pol} and K_d^{dNTP} values. A decrease in k_{pol}/K_d^{dNTP} associated with the deazapurine substitution would indicate that the position is involved in a crucial hydrogen bond. During correct base pair formation, the adenine to 1-deazaadenine substitution in both the incoming dNTP and template base resulted in a >1000-fold decrease in k_{pol}/K_d^{dNTP}, indicating that interstrand hydrogen bonds are important in correcting base pair formation. During formation of purine−purine mispairs, the k_{pol}/K_d^{dNTP} values for the insertion of dATP and dGTP opposite 7-deazaadenine and 7-deazaguanine were decreased >10-fold with respect to those of the unmodified nucleotides. In addition, the rate of incorporation of 1-deaza-dATP opposite guanine was decreased 5-fold. These results suggest that during mispair formation the newly forming base pair is in a Hoogsteen geometry with the incoming dNTP in the *anti* conformation and the template base in the *syn* conformation. These results indicate that Dpo4 holds the incoming dNTP in the normal *anti* conformation while allowing the template nucleotide to change conformations to allow reaction to occur. This result may be functionally relevant in the replication of damaged DNA in that the polymerase may allow the template to adopt multiple configurations.

DNA polymerases have been classified into six families based upon sequence similarities. In humans, the B-family polymerases α, δ, and ε are responsible for the high-fidelity DNA replication that occurs during S phase. Recent mechanistic and structural studies have led to the hypothesis that replicative polymerases achieve their high fidelity of nucleotide choice by selecting for the shape of the base pair (*1, 2*). These polymerases rapidly replicate undamaged nucleotides but stall when they encounter modified nucleotides. The stalled replication complex can be rescued by the translesion synthesis (TLS) polymerases. These low-fidelity polymerases consist of Y-family polymerases η, κ, and ι, B-family polymerase ζ, and perhaps A-family polymerases ν and θ. The low fidelity of these polymerases is the trade-off for the ability to rescue the stalled replication. Understanding the mechanistic and structural features that make bypass polymerases different from other polymerases is therefore important for understanding how DNA damage is converted into mutations and how health problems arising from the mutagenesis can be alleviated (*3*).

Sulfolobus solfataricus DNA polymerase IV (Dpo4)[1] is a thermostable Y-family polymerase that has been very useful as a model for bypass polymerases. It replicates DNA with low fidelity, incorporating an incorrect dNTP once every 1000−10000 replication events (*4*). Its kinetic mechanism is similar to those of many polymerases, including *Escherichia coli* DNA polymerase I (*5*) and T7 DNA polymerase (*6*), in that during correct base formation there is a rate-

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[1] Abbreviations: 1DA, 1-deazaadenine; 7DA, 7-deazaadenine; 7-DG, 7-deazaguanine; BF, large fragment of *Bacillus* DNA polymerase I; Dbh, dinB polymerase of *Sulfolobus acidocaldarius*; Dpo4, *S. solfataricus* DNA polymerase IV; Kf(exo$^-$), DNA polymerase I (Klenow fragment) with the proofreading exonuclease activity inactivated; pol, polymerase.

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limiting kinetic step after dNTP binding and prior to formation of a phosphodiester bond (*7*). Mismatches are produced with a decreased level of ground-state binding of the dNTP (2−10-fold) as well as a decreased rate of reaction (2−3 orders of magnitude) (*4*). In contrast, to correct base pair formation, the rate-limiting step for mispair formation is phosphodiester bond formation (*7*).

Dpo4 has been crystallized in a ternary complex with template−primer and incoming nucleotides, and both correctly matched pairs (*8, 9*) and mismatched pairs (*8–10*) as well as damaged DNA (*11–15*). The correct base pairs are found in the typical Watson−Crick orientation. The Dpo4 structures differ from replicative polymerases in that the active site is more open and more exposed to the solvent. Both the major and minor grooves of the DNA are more solvent-exposed and are perhaps more able to accommodate base pairs that differ from the standard Watson−Crick topology. A crystal structure of the attempted incorporation of dGTP opposite dG does not show a dGTP/dG base pair, but the dGTP is in a Watson−Crick hydrogen bonding complex with a dC 5′ to the template dG. The 3′-hydroxyl terminus, however, is far from the triphosphate, and reorganization must occur for reaction to occur (*9*). In the extension of a G/T mispair, the base pair has been found in a reverse wobble conformation in which the orientation of the deoxyribose moiety is reversed so that the 3′-OH group is not pointing toward the dNTP and is consequently an inactive conformation (*8, 10*).

The open active sites of the Y-family polymerases suggest that selection of the Watson−Crick geometry by protein−DNA interactions plays a lesser role in the selection of the correct dNTP fidelity than in replicative polymerases. Chemical studies with pol κ and η have indicated that interstrand hydrogen bonds play a larger role in the selection of the dNTP than for the replicative polymerases (*16, 17*). In addition, Dpo4 was found to use interstrand distances as a factor in base pair selection, but the discrimination for Watson−Crick distances was not as strong as for A-family polymerases (*18, 19*). Despite the increased importance of DNA−DNA interactions in fidelity, the polymerases may be able to impose structural constraints on the DNA. For example, pol ι induces the template base to be in the *syn* configuration, thereby presenting the Hoogsteen base pair face to the incoming dNTP.

In this work, we probe the importance of interstrand hydrogen bonding in the formation of Dpo4-catalyzed mispair formation. Toward this end, we employed the chemical approach of atomic substitution of the DNA. This kinetic technique allows us to identify critical interactions that occur along the reaction pathway; thus, we can probe the high-energy intermediates and transition states that may be inaccessible by X-ray crystallographic techniques.

EXPERIMENTAL PROCEDURES

Reagents. [^{32}P]ATP (6000 Ci/mmol) was purchased from Perkin-Elmer and T_4 polynucleotide kinase from USB, and purine nucleoside phosphoralyase and thymidine phosphorylase were purchased from Sigma-Aldrich. The dNTPs (ultrapure grade) were purchased from GE Healthcare, and the concentrations were determined by UV absorbance (*20*). The synthesis of 1-deaza-dA and derivatives as well as the oligodeoxynucleotide syntheses is described in the Supporting Information.

Polymerase Kinetics. The concentrations of oligodeoxynucleotides were determined from the absorbance at 260 nm, using the method of Borer (*21*) in which it was assumed that the spectroscopic properties of the modified nucleotides were identical to those of the unmodified nucleotides. The primer was ^{32}P-labeled with [γ-^{32}P]ATP and annealed with a 50% excess of the template as previously described (*22*). The DNA polymerase reactions were initiated by the addition of equal volumes of the dNTP in 50 mM Tris-HCl (pH 7.8) and 10 mM $MgCl_2$ to the DNA/enzyme solution in 50 mM Tris-HCl (pH 7.8), 60 mM NaCl, 0.1 mM EDTA, 5 mM DTT, and 100 μg/mL BSA at 37 °C. Rapid reactions were carried out with a KinTek-3 rapid quench instrument (KinTek Corp., Sandia, TX), but most reactions were performed by hand. The buffer during the reaction consisted of 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 30 mM NaCl, 50 μM EDTA, 2.5 mM DTT, and 50 μg/mL BSA. Typically, the DNA concentration was 25 nM and the Dpo4 concentration was 50 nM. The concentration of dNTPs varied from 0 to 5000 μM. The reactions were quenched by the addition of 300 mM EDTA.

Product Analysis by PAGE. The progress of the reaction was analyzed by denaturing PAGE in 15% acrylamide (19:1, acrylamide-*N*,*N*′-methylene bisacrylamide) and 7 M urea in 1× TBE buffer (0.089 M Tris, 0.089 M boric acid, and 0.002 M Na_2EDTA). The size of the gel was 40 cm × 33 cm × 0.4 cm and was run at 2000 V for 2−2.5 h. The radioactivity on the gel was visualized with a Molecular Imager FX Pro Plus PhosphorImager. The progress of the reaction was quantitated by dividing the total radioactivity in the product band(s) by the radioactivity in the product and reactant bands. Multiple product bands appeared when the incorrect dNTP was added to the reaction mixture.

Data Analysis. Data were fitted by nonlinear regression using Prism version 4 for Windows (GraphPad Software, San Diego, CA). Data from the reactions were fitted to eq 1

$$P = A(1 - e^{-kt}) \quad (1)$$

where P is the amount of product formed, A is the total amount of DNA reacted, and k is the first-order rate constant for incorporation of dNTP. The k values for these experiments were fitted to eq 2

$$k = k_{pol}[dNTP]/([dNTP] + K_d) \quad (2)$$

where k_{pol} is the maximum k of dNTP incorporation and K_d is the equilibrium dissociation constant for the interaction of dNTP with the polymerase−DNA complex.

RESULTS

Purine−purine mispairs can potentially form by the structures illustrated in Figure 1. These structures include conformations that were observed in duplex DNA [Figure 1, panels a (*23*), d (*24–27*), e (*27–30*), f (*31, 32*), g (*29, 33, 34*), and h (*35*)] as well as in the binding site of the large fragment of *Bacillus* DNA polymerase I (BF) [Figure 1, panels e and g (*29*)]. In addition, the A/A mispairs in panels b and c of Figure 1 were also considered because Figure 1b can form from a shift from Figure 1a, and Figure 1c is the A/A analogue of panels e and g of Figure 1. In

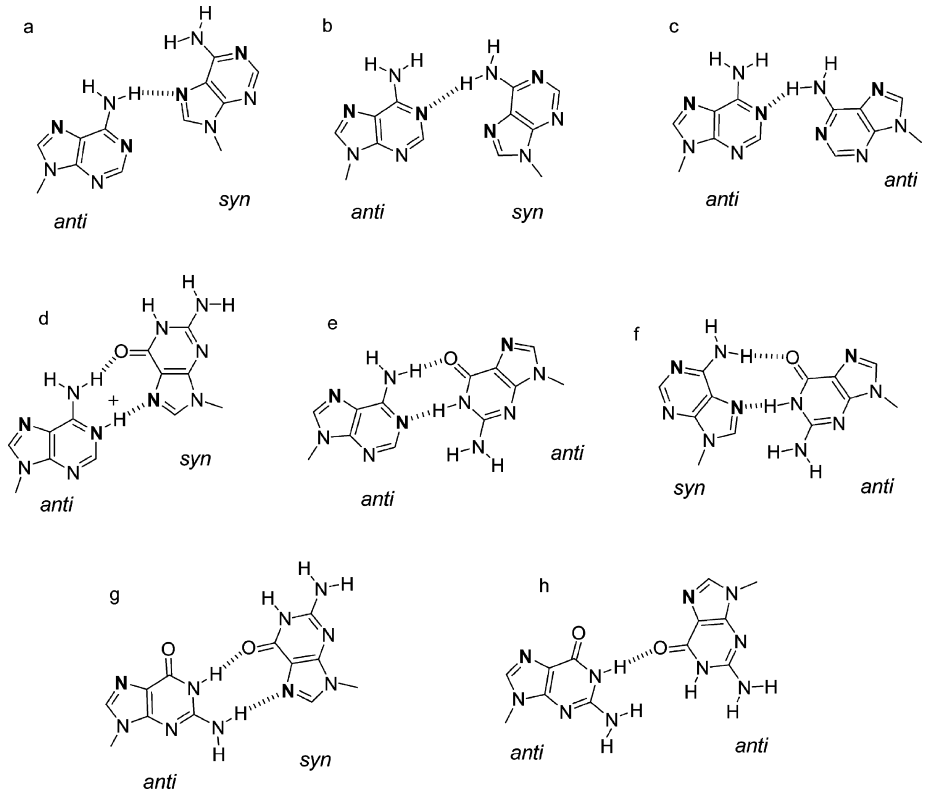

FIGURE 1: Potential structures of purine−purine base pairs: a (*23*), d (*24–27*), e (*27–30*), f (*31, 32*), g (*29, 33, 34*), and h (*35*).

panels c, e, and h of Figure 1, the orientations of the glycosidic bonds are both in the *anti* conformation, while in the other structures, the glycosidic bond of one purine has rotated to the *syn* conformation which allows Hoogsteen base pairing to occur. The interstrand distance of the Hoogsteen base pairs is Watson−Crick-like with distances between the carbons at the 1-position of the deoxyribose moieties of approximately 10.7 Å. The *anti*/*anti* conformation increases the distances between the 1-positions to ~12.3 Å.

The structures in Figure 1 differ in their interstrand hydrogen bonds. We used atomic substitution of the DNA, in which a nitrogen atom was replaced with a carbon and a proton, to probe interstrand hydrogen bonds. This approach was recently used to determine interstrand hydrogen bonding for DNA polymerase η (*17*), DNA polymerase ι (*36*), and Kf(exo$^-$) (*37*). If a hydrogen bond was important for phosphodiester bond formation, then we expect the rate of reaction (k_{pol}) to decrease. The decrease in rate can be due to loss of the hydrogen bond or to the steric effects of the proton not allowing certain conformations to occur.

1-Deaza-dA, 7-deaza-dA, and 7-deaza-dG were used as probes for interstrand hydrogen bonding during formation and extension of purine−purine mispairs. In these nucleoside analogues, the N to C substitution causes a number of changes in the molecule. The most drastic is the elimination of a hydrogen bond acceptor with the small increase in steric bulk at that position. All other heteroatoms would retain their hydrogen bonding character. We did not employ the 1-deaza-dG analogue, because the substitution would transform the carbonyl at the 6-position into a hydroxyl group. Thus, a change along the Watson−Crick hydrogen-bonding face could affect the Hoogsteen hydrogen-bonding face.

The sequences for the primer−template oligodeoxynucleotides are shown in Chart 1. **1** was employed to examine

Chart 1: Sequences of Oligodeoxynucleotide Substrates

1	5'- G C A C C G C A G A C G C A G	-3'
	3'- C G T G G C G T C T G C G T C X Y C A G C G T	-5'
2	5'- G C A C C G C A G A C G C A X	-3'
	3'- C G T G G C G T C T G C G T Y G T C A G C G T	-5'
3	5'- G C A C C G C A G A C G C A G X	-3'
	3'- C G T G G C G T C T G C G T C Y T C A G C G T	-5'

the formation, while **2** and **3** were used for the extension past purine−purine mispairs. Figure 1 shows the potential base pairing between the purine−purine mispairs with the interstrand hydrogen bonds. If a hydrogen bond was important in binding of the dNTP to the polymerase, then disruption of this bond should increase the dissociation constant (K_d) of the dNTP. If a hydrogen bond was important for phosphodiester bond formation, then the maximal rate of the reaction (k_{pol}) would decrease. This approach was recently used to assess interstrand hydrogen bonding for DNA polymerase η (*17*), DNA polymerase ι (*36*), and Kf(exo$^-$) (*37*).

The time course for the incorporation of a single nucleotide into oligodeoxynucleotides was determined with a DNA concentration of 25 nM and a Dpo4 concentration of 50 nM. The reactions went to completion, and the time courses followed pseudo-first-order kinetics and were fit to eq 1. Examples of these reaction time courses are shown in Figure S3 and S4 of the Supporting Information. The rates of reaction did not increase with 200 nM Dpo4 (data not shown), indicating that the DNA was completely bound by polymerase and that the reduced rates of reactions observed with mispair formation and extension with and without the deazapurines were not due to a decreased level of binding of the DNA to the polymerase. The single-turnover kinetics

Table 1: Effect of Nucleotide Modification on the Formation of Base Pairs[a]

dNTP	X	Y	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d (M^{-1} s^{-1})
T	A	T	3.2 ± 0.7	100 ± 27	(3.2 ± 1.1) × 10^4
T	1DA	T	0.0006 ± 0.00005	104 ± 30	6 ± 2
T	7DA	T	3 ± 0.9	178 ± 75	(1.7 ± 0.8) × 10^4
A	T	T	4.2 ± 0.9	187 ± 43	(2.2 ± 0.7) × 10^4
1DA	T	T	0.0040 ± 0.0001	200 ± 100	20 ± 10
7DA	T	T	3.1 ± 0.9	275 ± 103	(1.1 ± 0.5) × 10^4
C	G	T	3.5 ± 0.3	30 ± 10	(12 ± 4) × 10^4
C	7DG	T	3.25 ± 0.24	100 ± 32	(3.2 ± 1.0) × 10^4
G	C	T	5.7 ± 2	172 ± 30	(3.3 ± 1.3) × 10^4
7DG	C	T	4.25 ± 0.25	325 ± 56	(1.3 ± 0.2) × 10^4
A	A	A	0.0059 ± 0.0003	443 ± 44	13 ± 1
A	A	T	0.0078 ± 0.003	301 ± 201	26 ± 15
1DA	A	T	0.0213 ± 0.002	229 ± 49	93 ± 22
7DA	A	A	0.0043 ± 0.0004	844 ± 149	5.1 ± 1.0
7DA	A	T	0.0092 ± 0.004	168 ± 102	55 ± 25
A	1DA	T	0.0025 ± 0.001	187 ± 55	13 ± 6
A	7DA	T	0.0016 ± 0.0002	333 ± 222	4.7 ± 1.2
A	G	A	0.0041 ± 0.0003	531 ± 117	7.7 ± 1.7
A	G	T	0.020 ± 0.002	399 ± 215	50 ± 2
1DA	G	T	0.0052 ± 0.0025	475 ± 65	11 ± 4
7DA	G	A	0.0032 ± 0.0002	674 ± 72	4.7 ± 0.5
7DA	G	T	0.031 ± 0.007	210 ± 125	147 ± 73
A	7DG	T	0.00012 ± 0.00005	24 ± 2	4.9 ± 2.2
G	A	A	0.008 ± 0.001	831 ± 197	9.5 ± 2.6
G	A	T	0.005 ± 0.002	160 ± 65	29 ± 13
7DG	A	A	0.0023 ± 0.0004	305 ± 117	7.61 ± 3.2
G	1DA	T	0.0065 ± 0.0028	232 ± 37	28 ± 13
G	7DA	T	0.00028 ± 0.00001	146 ± 12	1.90 ± 0.16
G	G	A	0.00059 ± 0.00020	280 ± 121	2.1 ± 0.7
G	G	T	0.0041 ± 0.0014	255 ± 206	16 ± 4.7
7DG	G	A	0.00051 ± 0.00005	271 ± 71	1.87 ± 0.53
7DG	G	T	0.008 ± 0.001	440 ± 250	19 ± 4
G	7DG	T	0.00010 ± 0.00004	122 ± 14	0.84 ± 0.33
7DA	7DA	T	0.0011 ± 0.000065	94 ± 21	12 ± 3
7DA	7DG	T	0.00034 ± 0.000037	241 ± 77	1.4 ± 0.5
7DG	7DA	T	0.000076 ± 0.000003	97 ± 13	0.78 ± 0.11
7DG	7DG	T	0.000030 ± 0.000002	222 ± 36	0.13 ± 0.02

[a] The kinetic parameters were determined with 25 nM oligodeoxynucleotide **1** and 50 nM Dpo4 at 37 °C. The k_{pol}, K_d, and k_{pol}/K_d values are means ± the standard deviation of at least three experiments.

were used in favor of steady-state kinetics so that in the analyses of the data we could ignore the rate of dissociation of the DNA, the step that is rate-limiting in multiple turnovers of DNA polymerization (*38*). The first-order rate constants were plotted versus dNTP concentration, and the k_{pol} and K_d parameters were determined by fitting the data to eq 2. The resulting parameters are presented in Tables 1 and 2. In general, for the natural nucleotides, the decrease in the level of mispair formation was due to a 100−1000-fold decrease in k_{pol} and a 10−50-fold increase in K_d. The level of mispair extension was reduced due to a 100-fold decrease in k_{pol} and a 10-fold increase in K_d.

The results for the deaza substitutions during correct base pair formation are summarized in Figure 3, in which the ordinate is the k_{pol}/K_d of the reaction with the deazapurine divided by that of the unmodified reaction. A downward bar would indicate that the k_{pol}/K_d had decreased with the purine to deazapurine substitution. The solid bars show that the 7-deaza-dA and 7-deaza-dG substitutions in neither the dNTP nor the template affected the k_{pol}/K_d of the reactions. These results indicate that the 7-positions of the purines are not involved in any important DNA−DNA or DNA−protein interactions during correct base pair formation. In contrast, the white bars show that the rates of incorporation of 1-deaza-dATP opposite dT (Figure 3A) and dTTP opposite 1-deaza-dA (Figure 3B) were significantly reduced when compared with that of unmodified adenine. Significantly, the ~1000-fold decreases in k_{pol}/K_d are almost entirely due to decreases in k_{pol}, indicating that the loss of the interstrand hydrogen bond does not decrease the binding affinity for dTTP. The decreases in k_{pol} are similar to those observed with the

Table 2: Effect of Nucleotide Modification on the Extension of Purine−Purine Mispairs[a]

DNA	primer X	template Y	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d (M^{-1} s^{-1})
2	A	A	0.017 ± 0.004	135 ± 13	126 ± 32
2	1DA	A	0.006 ± 0.003	153 ± 21	39 ± 20
2	7DA	A	0.0042 ± 0.0001	201 ± 100	21 ± 10
2	A	G	0.021 ± 0.0070	138 ± 20	152 ± 55
2	1DA	G	0.017 ± 0.0050	156 ± 20	109 ± 35
2	7DA	G	0.013 ± 0.0030	104 ± 37	125 ± 53
2	G	A	0.017 ± 0.0100	150 ± 20	113 ± 68
2	7DG	A	0.044 ± 0.0140	149 ± 12	295 ± 97
2	G	G	0.012 ± 0.0015	149 ± 37	81 ± 22
2	7DG	G	0.0038 ± 0.0012	121 ± 13	31 ± 10
3	A	A	0.021 ± 0.0182	260 ± 8	80 ± 70
3	A	1DA	0.0057 ± 0.0007	299 ± 51	19 ± 4
3	A	7DA	0.0008 ± 0.0007	164 ± 62	5 ± 5
3	A	G	0.0076 ± 0.0007	261 ± 33	29 ± 4
3	7DA	G	0.0056 ± 0.0006	136 ± 24	41 ± 8
3	A	7DG	0.0038 ± 0.0016	169 ± 7	22 ± 10
3	G	A	0.0596 ± 0.0218	316 ± 133	189 ± 105
3	G	1DA	0.0360 ± 0.0060	603 ± 105	60 ± 14
3	G	7DA	0.0024 ± 0.0005	573 ± 287	4 ± 2
3	G	G	0.0131 ± 0.0039	579 ± 275	23 ± 13
3	G	7DG	0.0032 ± 0.0026	246 ± 149	11 ± 7

[a] The kinetic parameters were determined for the correct dNTP with 25 nM oligodeoxynucleotide and 50 nM Dpo4 at 37 °C. The k_{pol} and K_d values are means ± the standard deviation of three experiments. The error for the k_{pol}/K_d is based upon the propagation of errors (*43*).

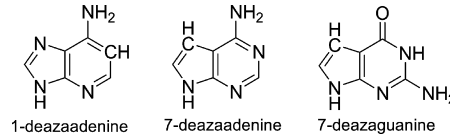

FIGURE 2: Structures of the deazapurines.

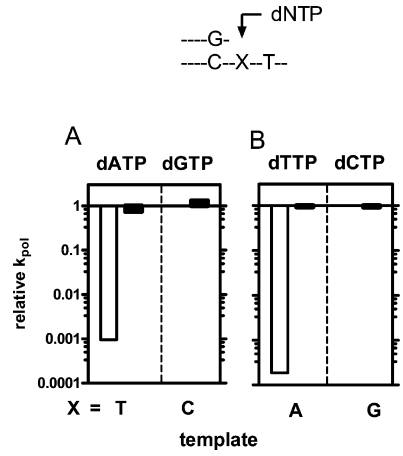

FIGURE 3: Effect of deaza substitutions on the formation of Watson−Crick base pairs. (A) Effect of the purine to deazapurine substitutions on the incorporation of dATP opposite dT and dGTP opposite dC. (B) Effect of the purine to deazapurine substitution on the incorporation of dTTP opposite dA and dCTP opposite dG. The *y*-axis is the k_{pol}/K_d of the deazapurine divided by the k_{pol}/K_d of the corresponding purine. The white bars represent the effect of the adenine to 1-deazaadenine substitution, while the black bars represent the effect of the adenine to 7-deazaadenine or guanine to 7-deazaguanine substitution.

thymine to difluorotoluene substitution with Kf(exo$^-$) and the *S. acidocaldarius* Dbh and Dpo4 polymerases (*39, 40*). Since the deazapurine substitution increases the activation energy controlling phosphodiester bond formation without affecting the ground-state binding of the dNTP, the loss of the hydrogen bond primarily affects the energy of the transition state involved in phosphoryl transfer.

Mispairs can form by two mechanisms, through the formation of non-Watson−Crick base pairs and through a slip-template mechanism in which the incoming dNTP pairs with the nucleotide in the +1 template position (Y in Table 1). The slip-template mechanism would result in a deletion mutation. This mechanism has been diagnosed by an increase in the rate of incorrect dNTP incorporation when the dNTP complement was placed at the +1 template position. This type of mechanism occurs readily with Dbh but is dependent on the sequence of the DNA. With Dbh, this mechanism appears to be sequence specific with increases in rate between 5- and 170-fold that are ascribed to the slip-template mechanism (*41*). This mechanism was also found with Dpo4, in which, depending on sequence, an increase of 42-fold in k_{pol}/K_d can be observed (*4*). We evaluated whether the slip-template mechanism occurred with the oligodeoxynucleotide sequence we employed using the oligodeoxynucleotides in Table 1 with a Y of dA or dT. The k_{pol}/K_d for insertion of dATP opposite A with an A in the +1 template position was 13 M^{-1} s^{-1}. When T was in the +1 position, the k_{pol}/K_d increased 2-fold to 27 M^{-1} s^{-1}. Similarly, the k_{pol}/K_d increased 6.5-fold during the incorporation of dATP opposite G when the +1 nucleotide was changed from A to T. The increases in k_{pol}/K_d are consistent with a slip-template mechanism, but the magnitudes are much smaller than those that have been observed for Dpo4 and Dbh (*4, 41*). We further evaluated the slip-template mechanism using 1-deaza-dATP. If dATP was inserted opposite A and/or G with T in the +1 template position via the slip-template mechanism, then 1-deaza-dATP should be inserted more slowly since it cannot form a Watson−Crick hydrogen bond complex with the T. The dATP to 1-deaza-dATP substitution with the AT template resulted in a 3.4-fold increase in k_{pol}/K_d, from 27 to 93 M^{-1} s^{-1}, which is clearly inconsistent with the slip-template mechanism. The 1-deaza-dATP substitution with the GT template resulted in a 4-fold decrease in k_{pol}/K_d, from 50 to 12 M^{-1} s^{-1}. While the decrease in k_{pol}/K_d is consistent with the Watson−Crick dATP/T base pair, the magnitude of the decrease, however, is orders of magnitude smaller than what we observed during the formation of the dATP/T base pair. Thus, we conclude that with our sequence context the primary mechanism of incorrect dNTP incorporation is not via the slip-template mechanism, but via mispair formation. The caveat to this analysis is that we have assumed that both the normal and slip-template dATP/dT base pairs would be similarly perturbed by the adenine to 1-deazaadenine substitution.

We probed the structure of the purine−purine mispair during replication with the oligodeoxynucleotide and deazapurine combination listed in Table 1. The effect of the deaza substitutions varied depending on the identity of the mispair. The results are presented in Table 1 and summarized in Figure 4, in which panel A shows the results of the experiments in which the dNTP was modified and panel B shows the modified purine was in the template. The largest

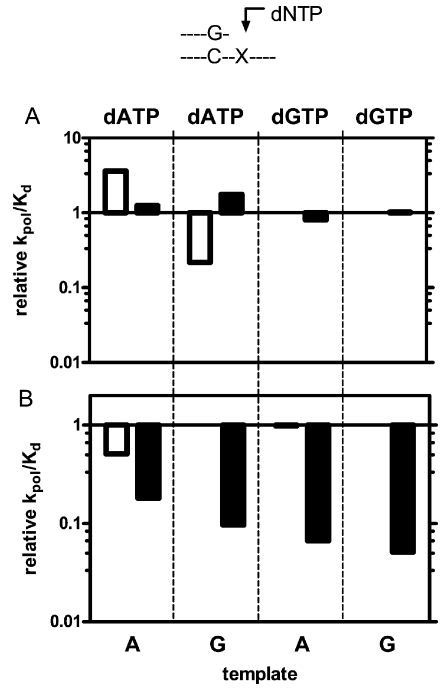

FIGURE 4: Effect of deaza substitutions on the formation of purine−purine mispairs. The dotted lines separate the four base pairs with the dNTP listed above and the template below the graphs. The *y*-axis is the relative k_{pol}/K_d of the reaction with the modified substrate divided by the unmodified substrate. The white bars represent the effect of the dA to 1-deaza-dA substitution, while the black bars represent effect of the substitution on the 7-position of either adenine or guanine. If the reaction was carried out with both A and T at the T + 1 position, the value represents an average of those experiments: (A) effect of the substitutions in the dNTP and (B) effect of the substitutions in the template strand.

decreases in k_{pol}/K_d are observed in panel B, in which the dA to 7-deaza-dA and dG to 7-deaza-dG substitutions are in the template. In general, these results suggest that the template nucleotides would be in the *syn* conformation with the Hoogsteen hydrogen-bonding face in contact with the base pair.

In the formation of the dA/dA base pair, the k_{pol}/K_d decreased 6-fold when 7-deaza-dA was substituted in the template. In contrast, the k_{pol}/K_d experienced only a modest increase or decrease, depending on the nucleotide at the +1 template position, with 7-deaza-dATP replacing dATP. The 1-deaza-dA substitution resulted in a 2-fold decrease in the amount of template and a 3-fold increase in the amount of dNTP. The 6-fold decrease in k_{pol}/K_d with 7-deaza-dA in the template is similar to that of the other mispairs in which the 7-deaza substitution on the template resulted in a decreased k_{pol}/K_d. It is consistent with the base pair structure in Figure 1a in which the dNTP is in the *anti* conformation while the template dA is in the *syn* conformation. However, the 6-fold decrease in k_{pol}/K_d is smaller than that obtained with the 7-deazapurine substitutions with the other mispairs. The alternative Hoogsteen base pair in Figure 1b is not supported by our results because we would have expected 1-deaza-dATP to be incorporated more slowly than dATP.

In the formation of the dATP/dG base pair, the k_{pol}/K_d was decreased 4-fold with the 1-deaza-dATP substitution and 10-fold with 7-deaza-dG in the template. The 7-deaza-dATP substitution caused a small increase in rate. These results are consistent with the structure in Figure 1d in which dATP

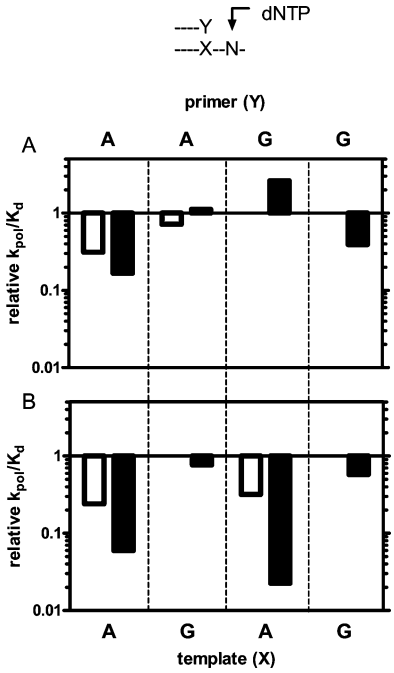

FIGURE 5: Effect of deaza substitutions on the extension of purine−purine mispairs. The dotted lines separate the four base pairs with the primer terminus (Y) listed above and the template (X) below the graph. The *y*-axis is the relative k_{pol}/K_d of the reaction in which the k_{pol}/K_d with the modified substrate is divided by the k_{pol}/K_d for the unmodified substrate. The white bars represent the effect of the dA to 1-deaza-dA substitution, while the black bars represent thr effect of the dA to 7-deaza-dA and dG to 7-deaza-dG substitutions: (A) effect of the substitutions in the primer strand and (B) effect of the substitutions in the template strand.

is in the *anti* conformation while the template dG is in the *syn* conformation.

In the formation of the dG/dA base pair, the 7-deaza-dA substitution in the template resulted in an 18-fold decrease in k_{pol}/K_d. The 1-deaza-dA substitution in the template and the incorporation of 7-deaza-dGTP resulted in virtually no change in rate. These results are consistent with the structure in Figure 1f in which dGTP is in the *anti* conformation while the template dA is in the *syn* conformation.

In the formation of the dG/dG base pair, the substitution of 7-deaza-dG in the template resulted in a 20-fold reduction in k_{pol}/K_d. The corresponding substitution in the dNTP did not change the rate of reaction. These results indicate that the N7-position of dG in the template is involved in an important hydrogen bond and is consistent with the base pair illustrated in Figure 1g in which dNTP is in the *anti* conformation while the template is in the *syn* conformation.

The structure of the base pair during extension past a mispair was examined with oligodeoxynucleotides **2** and **3** with the nucleotide pairs listed in Table 2. The results are summarized in Figure 5. Dpo4 does discriminate against the extension of purine−purine mispairs, but to a lesser extent than for mispair formation. Comparison of the kinetic constants for oligodeoxynucleotides **1** and **2** shows a 360-fold decrease in k_{pol}/K_d for mispair extension. This discrimination is 20-fold weaker than what we observed for KF^-, which shows a 6000-fold decrease in k_{pol}/K_d for mispair extension (*37*). The deaza substitutions produced variable effects. The largest effects that we observed occurred with the 7-deaza-dA substitutions in the template of the A/A and G/A mispairs. These effects would be consistent with the structures in panels a and f of Figure 1 in which the primer terminus would be in the *anti* conformation while the template in the *syn* conformation. This was also the configuration predicted for the formation of the mispairs. If this structure occurred generally, we would expect that 7-deaza-dG in the template would decrease the rate of reaction. However, in the extension of dG/dA amd dG/dG base pairs, the 7-deaza-dG substitution in the primer produced only small decreases in k_{pol}/K_d.

DISCUSSION

Y-Family polymerases are essential in the replication of damaged DNA but replicate undamaged DNA with reduced fidelity, two characteristics that are linked by the catalytic properties of theses enzymes. Structural studies have shown that the binding and active sites are more open than in the so-called "replicative" polymerases. It is thought that having a large binding site allows the damaged nucleotide, which is typically larger, to fit into the Y-family polymerase binding sites more effectively than in the replicative polymerase. However, the fundamental question of how the Y-family polymerases replicate DNA damage remains unanswered. Do the large binding sites allow the DNA−DNA interactions to control fidelity? Or do the polymerases impose specificity upon the reactions by adapting to different substrates by shifting side chains and backbones to impose polymerase specific structural constraints on the base pairs? Structural studies alone cannot answer these issues adequately for several reasons. First, crystallography captures stable structures that may not be along the reaction pathway. This may be especially true for the formation of mispairs and the replication of damaged DNA which occur slowly and may involve formation of stable nonproductive complexes. Second, the rates of reaction are controlled by the free energy differences between the ground-state intermediates and the transition states. While the ground-state intermediates can potentially be observed by X-ray crystallography experiments, the transition states cannot. Third, the resolution of the X-ray structures is low compared to the known sensitivity of steric effects.

The open active sites of the Y-family polymerases indicate that there are many fewer interactions between the protein and DNA that would select for Watson−Crick base pairs. Chemical studies with pol κ and η have indicated that interstrand hydrogen bonds play a larger role in the selection of the dNTP than for the replicative polymerases (*16, 17*). The question that we probed in this study is whether despite the open active site, Dpo4 imparts any geometric constraints on the formation of base pairs. Toward this end, we examined the base pair structure during formation of purine−purine mispairs. The structures in Figure 1 show the array of possible structures that may occur during mispair formation. These structures were probed using atomic substitution of the DNA. In this approach, a single heteroatom on the nucleic acid is changed and the kinetics are measured. If the heteroatom was involved in a crucial interaction, then the rate of reaction should decrease. This approach has an advantage over X-ray crystallography in that critical interactions that are identified are along the reaction pathway. It can also be determined whether these interactions are

involved in ground-state binding or in transition-state stabilization.

These results suggest that Dpo4 catalyzes the formation of purine−purine mispairs via Hoogsteen base pairs in which the incoming dNTP is in the normal *anti* conformation while the template nucleotide flips to the *syn* conformation. While the *anti*/*anti* configurations illustrated in panels c, e, and h of Figure 1 would allow the nucleotides to retain their normal conformation within the DNA, the interstrand distances would increase. In contrast, the Hoogsteen base pair scheme allows the DNA to retain Watson−Crick-like distances between the strands. DNA polymerases are sensitive to the interstrand distances, as measured by the glycosidic C1′−C1′ distance. Both Kf(exo$^-$) and Dpo4 have similar optimal interstrand distances (*18, 19, 42*). However, while Kf(exo$^-$) demonstrates a strict distance preference, Dpo4 reactivity is much less dependent on C1′−C1′ distances. Although Dpo4 has a reduced bias against an increase in interstrand distance as compared with Kf(exo$^-$) (*19, 42*), the polymerase prefers to form mispairs via the Hoogsteen base pair that retains the Watson−Crick-like interstrand distance. In addition, Dpo4 prefers a specific Hoogsteen geometry in which the template is in the *anti* conformation. If the active site was an unstructured active site that preferred Watson−Crick interstrand distances, then the Hoogsteen base pair could be formed with either geometry. Clearly, our results implicate a *syn* template and *anti* dNTP geometry.

We previously found that Kf(exo$^-$) also formed purine−purine mispairs via the Hoogsteen base pair geometry (*37*). However, Kf(exo$^-$) and Dpo4 differ in the orientation of the base pair. With Kf(exo$^-$), the dNTP is the nucleotide that rotates to the *syn* conformation while the template is held in the normal *anti* conformation. Another difference between Dpo4 and Kf(exo$^-$) is the effect that the deaza substitution has on the dA(*anti*)/dG(*syn*) base pair (Figure 1d). This Hoogsteen base pair is unique in that it is protonated. With Kf(exo$^-$), in the incorporation of dGTP opposite dA, the 7-deaza-dGTP substitution did not result in a decrease in rate. This we rationalized was due to the importance of geometry versus hydrogen bonding, in that the substitution would disrupt the protonated hydrogen bond but should not distort the geometry of the base pair. In contrast, with Dpo4, both the 1-deaza-dA and 7-deaza-dG substitutions resulted in decreased rates of reaction. From these results, we conclude that the hydrogen bond in itself is important for the correct orientation of the incoming dNTP. This is consistent with previous reports of Y-family polymerases in which interstrand hydrogen bonds play a larger role in the selection of the dNTP than for the replicative polymerases (*16, 17*).

The mechanism by which Dpo4 selects the dNTP(*anti*)/template(*syn*) configuration is not known. Presumably, the polymerase initially holds the template in the *anti* conformation. However, when reaction does not occur, the template nucleotide adopts the *syn* conformation for reaction to occur. The mechanism of the reorientation of the template is unknown. The rate of dissociation of the Dpo4−DNA complex is sufficiently rapid (*7*) to allow for the dissociation and rebinding of the DNA, but the open binding site of the polymerase may allow for reorientation to occur while it is bound to the polymerase. The ability of the template to adopt non-Watson−Crick structures may also play a role in the replication of carcinogen-damaged DNA. The flexibility that the polymerase has with the template positioning may enable reaction.

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SUPPORTING INFORMATION AVAILABLE

Synthesis and characterization of the phosphoramidite for 1-deazaadenine and examples of kinetic analysis of the DNA polymerase experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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