Klenow Fragment−DNA Interaction Required for the Incorporation of Nucleotides Opposite Guanine and O^6-Methylguanine†

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ABSTRACT: A mechanism by which the Klenow fragment of DNA polymerase I monitors the geometry of the base pairs may involve hydrogen bonds between the polymerase and the minor groove of the nascent base pair. The involvement of the 3-position of guanine in the template strand was examined by synthesizing oligodeoxynucleotides containing guanine and 3-deazaguanine and comparing the steady-state kinetics of the incorporation of all four dNTPs. The V_{max}/K_m decreased a significant amount (170-fold) only when dCTP was the co-substrate suggesting that a hydrogen bond exists only when the correct base pair is being replicated. This approach was also used to examine how the Klenow fragment interacts with the 3-position of the mutagenic base O^6-methylguanine (O^6mG). The V_{max}/K_m for the incorporation of dTTP opposite O^6-methyl-3-deazaguanine (O^6m3DG) was 1700-fold less than opposite O^6mG. In contrast, a small 6-fold increase in V_{max}/K_m occurred for the incorporation of dCTP opposite O^6m3DG relative to O^6mG. This result suggests that the hydrogen bond between the Klenow fragment and O^6mG is more important in the incorporation of dTTP opposite O^6mG and may contribute to the mutagenicity of O^6mG.

Accurate DNA replication during synthesis and repair is essential in maintaining the integrity of the genome. Three processes contribute to the overall fidelity of DNA replication: the polymerization reaction, proofreading exonuclease activity, and post-replication mismatch repair. The accuracy in the polymerization step is on the order of 1 error in 10^3−10^5 base pairs depending on the polymerase (*1*). The energy difference between correct and incorrect base pairs is not enough to account for the accuracy in the polymerization reaction (*2*). The polymerase may enhance the fidelity of replication by checking the geometry of the bases during synthesis (*3*). The single active site can process all four possible Watson−Crick base pairs because they have very similar geometry while mismatches have very different geometry (*4*, *5*).

Interactions between the polymerase and the minor groove of the nascent base pair have been observed in X-ray crystallographic studies (*6*, *7*). In a ternary crystal structure with polymerase β, DNA, and ddCTP,[1] hydrogen bonds were detected between the N^3-position of guanine in the template and Arg283 and between the O^2-position of ddCTP and Asn279 (*6*). In a binary crystal structure with *Taq* DNA polymerase and blunt-end duplex DNA, hydrogen bonds were observed between the *N*3-position of guanine in the template and Arg746 and between the O^2-position of cytosine and Gln754 (*7*). Arg283 was found to be important in the function of polymerase β by site-directed mutagenesis of this residue to Lys, Leu, and Ala (*8*, *9*). No evidence, however, was found in site-directed mutagenesis experiments suggesting that Asn 279 of polymerase β was crucial for replication (*8*).

The importance of a hydrogen bond to the N^3-position of a purine in the template at the nascent base pair was also found in experiments using modified nucleotides. 2′-Deoxyxanthosine or 2′-deoxy-7-deazaxanthosine can form a Watson−Crick base pair with 2,4-diaminopyrimidine. However, 2′-deoxyxanthosine and 2′-deoxy-7-deazaxanthosine have a protonated nitrogen at the 3-position, which cannot act as a hydrogen bond acceptor. Polymerases α, ϵ, and the Klenow fragment were unable to incorporate 2,4-diaminopyrimidine triphosphate opposite either 2′-deoxyxanthosine or 2′-deoxy-7-deazaxanthosine (*10*).

Modification of DNA, such as the methylation of the 6-position of guanine, can lead to mutations (*11*−*13*). Although O^6mG−cytosine base pairs are more stable than O^6mG−thymine base pairs (*14*), thymine is inserted opposite O^6-methylguanine (O^6mG) more often than cytosine. The relative ratio of incorporation of thymine to cytosine opposite O^6mG depends on the polymerase and the sequence (*15*). dTTP may be a better substrate than dCTP because dTTP can form a Watson−Crick-like base pair complex with O^6-mG at physiological pH (*16*). In order for the O^6mG−dCTP complex to be Watson−Crick-like it must be protonated (*16*, *17*). Thus, at neutral pH, cytosine is inserted opposite O^6-mG via a wobble conformation while thymine is incorporated via a Watson−Crick-like conformation (*18*).

The minor groove interactions that favor the formation of Watson−Crick base pairs may also be involved in the preferential incorporation of thymine opposite O^6mG. The incorporation of thymine opposite O^6mG, however, is as slow as the misincorporation of thymine opposite guanine (*19*). Even though the dTTP−O^6mG complex is more Watson−Crick-like than the dCTP−O^6mG complex, it is still a poor substrate for the Klenow fragment (*19*). The dTTP−O^6mG

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[1] Abbreviations: BSA, bovine serum albumin; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; 3-deazaguanine, 3DG; DTT, dithiothreitol; dNTP, 2′-deoxynucleotide 5′-triphosphate; Kf (exo⁻), Klenow fragment with the exonuclease proofreading activity inactivated; O^6mG, O^6-methylguanine; O^6m3DG; O^6-methyl-3-deazaguanine, PAGE, polyacrylamide gel electrophoresis; *Taq*, *Thermus aquatucus*.

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complex may be as distorted from ideal Watson−Crick geometry, and interaction between the polymerase and the 3-position of O^6mG may not exist.

To examine the importance of a hydrogen bond from the polymerase to the 3-position of guanine and O^6mG, oligodeoxynucleotides containing guanine, O^6mG, 3-deazaguanine (3DG), and O^6-methyl-3-deazaguanine (O^6m3DG) were synthesized. The steady-state kinetics of the incorporation of the four native dNTPs were measured.

EXPERIMENTAL PROCEDURES

General. [^{32}P]ATP was purchased from Amersham at 6000 Ci/mmol. T_4 polynucleotide kinase and the Klenow fragment of *Escherichia coli* DNA polymerase I with the proofreading exonuclease inactivated (Kf (exo$^-$)) were obtained from USB. The dNTPs (ultrapure grade) were purchased from Pharmacia, and the concentrations were determined by UV absorbance (*20*). The oligonucleotides containing O^6mG and O^6m3DG were synthesized and characterized similarly to that previously described (*21*, *22*).

3-Deaza-2′-deoxy-N^2-phenoxyacetylguanine. The exocyclic amino group of 3-deaza-2′-deoxyguanosine (*21*) was protected with the phenoxyacetyl group via the transient protection method (*23*). ^{1}H NMR (DMSO-d_6) δ 11.4 (1H, br s, NH), 10.25 (1H, s, NH), 8.32 (1H, s, 8-deazaguanyl), 7.35 (2H, t, 2,6-phenyl), 7.05 (4H, m, 3,4,5-phenyl and 3-deazaguanyl), 6.16 (1H, t, 1-deoxyribosyl), 4.75 (2H, s, O-CH_2-CO), 4.35 (1H, m, 3-deoxyribosyl), 3.86 (1H, m, 4-deoxyribosyl), 3.51 (2H, d/d/d, 5-deoxyribosyl), 2.56 (1H, m, 2-deoxyribosyl), 2.33 (1H, m, 2-deoxyribosyl).

3-Deaza-2′-deoxy-5′-O-(4,4′-dimethoxytrityl)-N^2-phenoxyacetylguanine. The N^2-protected nucleoside was tritylated at the 5′-position with 4,4′-dimethoxytrityl chloride as described (*24*). ^{1}H NMR ($CDCl_3$) δ 7.81 (1H, s, 8-deazaguanyl), 7.2−6.6 (19H, m, trityl, phenyl, and 3-deazaguanyl), 6.09 (1H, t, 1-deoxyribosyl), 4.62 (2H, s, O−CH_2−CO), 4.50 (1H, m, 3-deoxyribosyl), 4.10 (1H, m, 4-deoxyribosyl), 3.60 (6H, s, methoxy), 3.17 (2H, m, 5-deoxyribosyl), 2.56 (1H, m, 2-deoxyribosyl), 2.45 (1H, m, 2-deoxyribosyl).

3′-O-[(2-Cyanoethyl)-N,N-diisopropylphosphoramidyl]-3-deaza-2′-deoxy-5′-O-(4,4′-dimethoxytrityl)-N^2-phenoxyacetylguanine. 3-Deaza-2′-deoxy-5′-(4,4′-dimethoxytrityl)-N^2-phenoxyacetylguanine (140 mg, 0.2 mmol) was reacted with 56 μL of 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite (0.25 mmol) and 70 μL of *N*,*N*-diisopropyl-*N*-ethylamine (0.4 mmol) in 6 mL of methylene chloride at room temperature for 20 min. The reaction mixture was washed with 5% $NaHCO_3$, dried over Na_2SO_4, and applied to silica column which was eluted with 5% methanol, 1% triethylamine in chloroform. The product was isolated in 80% yield. ^{1}H NMR ($CDCl_3$) δ 7.86 (1H, d, 8-deazaguanyl), 7.4−6.7 (19H, m, trityl, phenyl, and 3-deazaguanyl), 6.11 (1H, m, 1-deoxyribosyl), 4.72 (2H, s, O−CH_2−CO), 4.62 (1H, m, 3-deoxyribosyl), 4.22 (1H, m, 4-deoxyribosyl), 3.72 (6H, s, methoxy), 3.6 (4H, m, C$\underline{H}$(CH_3)$_2$ and O$C\underline{H}_2CH_2$CN), 3.35 (2H, m, 5-deoxyribosyl), 2.5 (4H, m, 2-deoxyribosyl and $OCH_2C\underline{H}_2$CN), 1.2−1.07 (12H, m, methyl). The assignments were made by comparison with starting material and through decoupling and COSY experiments.

The oligodeoxynucleotides, synthesized by standard solid phase phosphoramidite chemistry, include the primer and the template strands of the sequences described in Table 1. The templates were synthesized in which X was G, 3DG, O^6-mG, and O^6m3DG. The sequences were chosen to alternate the nucleotides but to keep a higher CG content so that the template−primer would stay annealed at a reasonable temperature and that the primer would anneal to the template in the correct position. The concentrations of oligodeoxynucleotides were determined from the absorbance at 260 nm, using an ϵ of 115 mM^{-1} cm^{-1} for the primer and 172 mM^{-1} cm^{-1} for the template (*25*). The primer was ^{32}P-labeled with [γ-^{32}P]ATP in a reaction catalyzed by T_4 polynucleotide kinase. The oligomer was separated from low molecular weight impurities with a spin column (Bio-Gel P6), and the primer was annealed with a 10% excess of the template as previously described (*22*).

Reaction with DNA Polymerase. The polymerase was added to a solution containing the ^{32}P-labeled oligodeoxynucleotide duplex and buffer, adjusted to the final conditions of 100 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 200 μg/mL BSA, pH 8.0. The reaction was initiated by the addition of 3 μL of dNTP in water to 3 μL of DNA−enzyme solution at 37 °C. The composition of the buffer during the reaction was 50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM DTT, 100 μg/mL BSA. The concentration of the primer was 106 nM and the template was 117 nM. The polymerase concentrations varied from 0.01 to 5 units/mL. The concentration of dNTPs varied from 0 to 1 mM. The reactions were quenched by the addition of 6 μL of 100 mM EDTA in 95% formamide. The progress of the reaction was analyzed by denaturing PAGE in 20% acrylamide (19:1, acrylamide:*N*,*N*′-methylene bisacrylamide), 7 M urea in 1× TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M Na_2EDTA). The size of the gel was 40 × 33 × 0.4 cm and was run at 2500 V for 2−2.5 h. The radioactivity on the gel was determined with a Bio-Rad GS 250 Molecular Imager.

RESULTS

The interaction between the Klenow fragment of DNA polymerase I of *E. coli* and the 3-position of guanine and O^6mG during replication was examined using analogs of the base in which the nitrogen at the 3-position was replaced by carbon. This modification eliminates the possibility of a hydrogen bond with that position. If a hydrogen bond was important then the rate of reaction with the deazaguanine will be much less than the native substrate.

The kinetics of the insertion of dATP, dCTP, dGTP, and dTTP opposite the G, 3DG, O^6mG, and O^6m3DG catalyzed by Kf (exo$^-$) were followed by PAGE. Initial rates were determined in reactions in which the extent of reaction was less than 20% complete. The kinetics, in all cases except in the incorporation of dCTP opposite 3DG, were found to be consistent with simple Michaelis−Menten kinetics illustrated by eq 1 and 2, in which S is the dNTP, E is the polymerase, DNA_n is the substrate oligodeoxynucleotide, and DNA_{n+1} is the oligodeoxynucleotide product. The kinetic parameters were determined by fitting the data to eqn 2 with the nonlinear least-squares curve-fitting routine in SigmaPlot.

$$\mathrm{E{-}DNA}_n + \mathrm{S} \rightleftharpoons \mathrm{E{-}DNA}_n\mathrm{{-}S} \rightarrow \mathrm{E{-}DNA}_{n+1} \quad (1)$$

$$v_o = \frac{V_{max}[\mathrm{S}]_o}{K_m + [\mathrm{S}]_o} \quad (2)$$

$$v_o = \frac{V_{max1}[S]_o}{K_{m1} + [S]_o} + \frac{V_{max2}[S]_o}{K_{m2} + [S]_o} \quad (3)$$

The v_o vs $[S]_o$ plot for the incorporation of dCTP opposite 3DG was not a simple hyperbolic plot (Figure 1). At low concentrations the plot appeared hyperbolic, but the initial rate continued to rise as the dCTP concentration increased. There appeared to be high and low K_m reactions with dCTP. The data were fit to both eq 2 and 3. Analysis of the data with eq 2 gave a V_{max} of 18 ± 2 pmol/min/unit polymerase and a K_m of 110 ± 40 μM. The kinetic parameters from eq 3 are presented in Table 1. Figure 1 presents the data with the theoretical curve for eq 2 and 3. The curve derived from eq 3 fits the data much better than eq 2.

The kinetic parameters for all the reactions are shown in Table 1. dCTP was incorporated opposite guanine about 16 000 times more frequently than the other nucleotides mainly due to a decreased K_m parameter. This result is consistent with that previously found for the Klenow fragment (*19*, *26*). When 3DG replaced guanine in the template, the V_{max}/K_m for the incorporation of dCTP decreased about 170-fold. The substitution of 3DG for G did not significantly affect the rate of incorporation for the other nucleotides.

In the incorporation of dCTP opposite 3DG, as indicated by Figure 1, both high and low K_m reactions were observed. The low K_m reaction had a V_{max} 10-fold less than that of dCTP opposite G, while the high K_m reaction had a V_{max} equal to the V_{max} of the insertion of dCTP opposite G. The high K_m was approximately equal to that of the other nucleotides opposite G and 3DG. The low K_m was 10-fold greater than the incorporation of dCTP opposite G.

Replacing G with 3DG did not significantly affect the binding of the DNA to the polymerase. The K_m^{DNA} was obtained by reaction of Kf (exo$^-$) with dCTP (50 μM) in the presence of 0−50 nM oligodeoxynucleotide duplex. As illustrated in Figure 2, the K_m^{DNA} did not change when the G was replaced with 3DG. The K_m^{DNA} with guanine in the DNA is 10 ± 4 nM, while that for 3DG is 16 ± 4 nM. These values are not very different for the 5 nM K_D of the DNA found in pre-steady-state experiments (*27*).

In the incorporation opposite O^6mG, the V_{max}/K_m of dTTP was four times larger than that of dCTP. dATP and dGTP were incorporated at least 10 times slower than dCTP. The relative rate of the incorporation opposite O^6mG was primarily dependent on the V_{max} parameter. When O^6mG was replaced by O^6m3DG, the V_{max}/K_m for dTTP was decreased 1400-fold due to a decrease in V_{max}. The V_{max}/K_m for the incorporation of dCTP actually rose by a factor of 6. The decrease in the V_{max} of 500-fold was countered by a 2900-fold decrease in K_m. The V_{max}/K_m for the incorporation of dATP dropped 10-fold due to a decrease in V_{max}. The V_{max}/K_m for the insertion of dGTP increased 5-fold.

DISCUSSION

The overall structures of polymerases, which have been crystallized, are similar (*6*, *7*, *28*−*30*). The structure has been described as a hand with palm, finger, and thumb domains (*28*). Conserved carboxylate containing amino acids, which bind to the essential divalent metal cations, are located on the palm domain. DNA binds to the enzyme

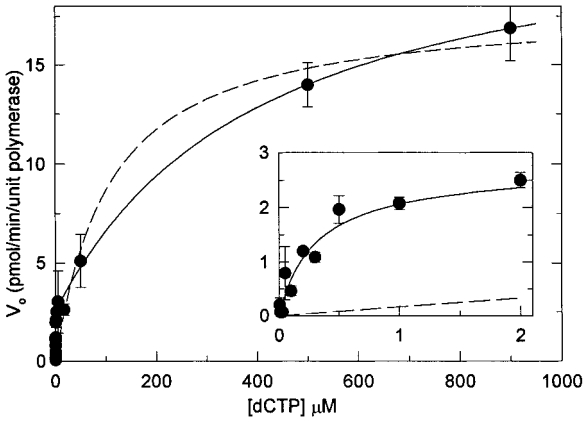

FIGURE 1: Initial rate of insertion of dCTP opposite templates containing 3DG. The concentration of Kf (exo$^-$) was 1 unit/mL, and DNA was 106 nM. Each point is an average of 3−5 determinations, and the error bars represent the standard deviations. The lines represent the theoretical curves calculated from the experimental points fitted to eq 2(---) and eq 3 (—).

along the palm and in between the thumb and finger domains. When the substrates bind to the polymerase, the thumb domain closes down over the DNA and dNTP (*6*).

The Klenow fragment (*29*), *Taq* polymerase (*7*), and polymerase β (*6*) have an arginine residue on the thumb domain which interacts with the N^3-position of guanine in the template at the nascent base pair. Since all four Watson−Crick base pairs have a similar geometry, this hydrogen bond may also exist between the Arg and the 3-position of adenine and the O^2-positions of cytosine and thymine. The formation of the hydrogen bond between the Arg and the minor groove of the DNA may be a general mechanism by which the polymerase checks the geometry of the newly forming base pair.

The hydrogen bond between this arginine residue and the 3-position of guanine may be crucial in the conformational change in which the thumb domain closes down over the DNA. The movement of the thumb domain may be the rate-limiting conformational change observed in pre-steady-state kinetics (see Figure 3) (*1*, *31*, *32*). When the arginine is replaced by alanine, mutant polymerase β (R283A), 9-base/11-base double-stranded DNA duplex and ddTTP crystallize in an open conformation in which the thumb domain is positioned as in the unbound protein (*8*). This mutant polymerase β is less active than the wild-type (*8*, *9*) and has a lower fidelity (*33*).

We have investigated the interaction between Arg841 of Kf (exo$^-$) and the 3-position of guanine using 3DG, an analog of G in which the nitrogen, at the 3-position is replaced by CH. The nitrogen can act as a hydrogen bond acceptor from arginine, but the CH group cannot participate in a hydrogen bond. The V_{max}/K_m for the reaction with dCTP decreased 170-fold when the nitrogen at the 3-position was replaced by carbon. This substitution did not influence the rate of formation of mismatches to a significant extent (0−5-fold decreases). These results are consistent with those obtained with wild-type polymerase β and the R283A mutant (*33*). Compared to wild-type, correct incorporations catalyzed by the R283A mutant were decreased 200−600 fold, while mispair formation was also decreased, but to a lesser extent (2−200-fold). In particular with guanine in the template, incorporation of dCTP was decreased 224-fold, dTTP 12-fold, dGTP 2-fold, and dATP 2-fold (*33*). In the

Table 1. Kinetic Parameters for the Insertion of dNTP Opposite Analogs of Guanine[a]

Template 3'- G C A G C T G C A G C T X C T A G T -5'

Primer 5'- C G T C G A C G T C G A -3'

dNTP ↑

template	kinetic parameter[b]	substrate dATP	dCTP		dGTP	dTTP
guanine	V_{max}	0.42 ± 0.03	22 ± 1		7 ± 1	8.0 ± 0.7
	K_m	118 ± 42	0.014 ± 0.003		68 ± 4	77 ± 22
	V_{max}/K_m	0.0046 ± 0.0004	1600 ± 320		0.104 ± 0.004	0.10 ± 0.02
3DG	V_{max}	0.39 ± 0.03	2.4 ± 0.3	21 ± 2	3.0 ± 0.2	3.3 ± 0.2
	K_m	85 ± 42	0.25 ± 0.08	400 ± 90	150 ± 25	89 ± 19
	V_{max}/K_m	0.0046 ± 0.0010	9.6 ± 3.1	0.053 ± 0.012	0.020 ± 0.002	0.037 ± 0.006
O^6mG	V_{max}	1.4 ± 0.2	31 ± 2		1.3 ± 0.2	96 ± 2
	K_m	74 ± 24	104 ± 14		45 ± 9	86 ± 12
	V_{max}/K_m	0.019 ± 0.003	0.3 ± 0.03		0.027 ± 0.003	1.1 ± 0.1
O^6m3DG	V_{max}	0.14 ± 0.01	0.060 ± 0.003		2.1 ± 0.1	0.098 ± 0.014
	K_m	53 ± 13	0.036 ± 0.012		24 ± 7	124 ± 49
	V_{max}/K_m	0.0026 ± 0.0005	1.7 ± 0.5		0.087 ± 0.016	0.00079 ± 0.00021

[a] Reaction was carried out in 50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM DTT, 0.1 mg/mL BSA, pH 8.0. The concentration of the primer was 106 nM. The concentration of the template was 117 nM. The concentration of the polymerase was different for each dNTP−template pair as described in the text. The error is the standard error. [b] The units are V_{max} (pmol/min/unit polymerase), K_m (μM), V_{max}/K_m (min^{-1} (unit polymerase/μL)$^{-1}$).

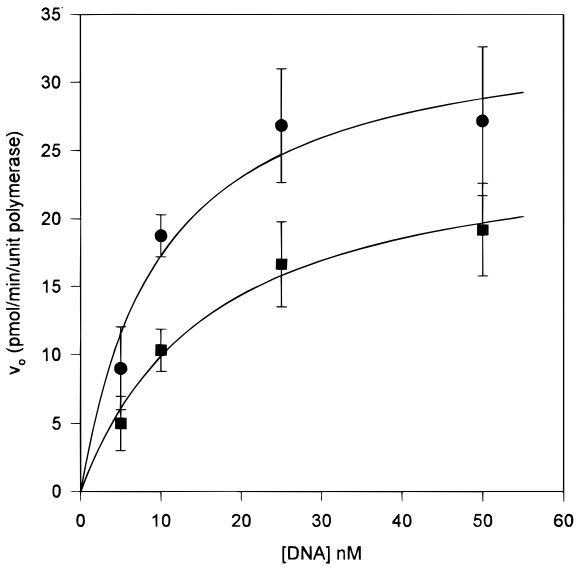

FIGURE 2: Initial rate for the incorporation of dCTP opposite G (●) and 3DG (■) at various DNA concentrations. The concentration of Kf (exo$^-$) was 1 unit/mL, and dCTP was 50 μM. Each point is an average of 3 determinations, and the error bars represent the standard deviations. The lines represent the theoretical curves calculated from the experimental points fitted to eq 2.

present experiments with Kf (exo$^-$), replacing guanine with 3-deazaguanine produced very similar changes in rate. The incorporation of dCTP was decreased 170-fold, dTTP 3-fold, dGTP 5-fold, and dATP no change in rate.

The disruption of the hydrogen bond between Arg841 and the 3-position of guanine can decrease V_{max}/K_m by slowing the rate-determining step. The rate-determining step of the incorporation of the correct dNTP is the conformational change prior to the formation of the phosphodiester bond (*31*). This conformational change has been suggested to be the thumb domain closing down over the DNA and dNTP. Since Arg841 is in the thumb domain, the hydrogen bond between Arg841 and the DNA may play a crucial role in the movement of the thumb domain over the DNA. Therefore eliminating this hydrogen bond may decrease the rate of the conformational change.

Alternatively, elimination of this hydrogen bond may decrease the V_{max}/K_m by slowing down a different step and thereby making that step rate limiting. Two steps which may be affected are the formation of the phosphodiester bond and the subsequent relaxation of the conformation (see Figure 3). Phosphodiester bond formation is rate limiting in the creation of mispairs (*34*), while the relaxation of the conformation is the rate-determining step in the incorporation of dCTP and dTTP opposite O^6mG (*35*).

As in the first conformational change, the hydrogen bond between Arg841 and the 3-position of guanine may be critical for the thumb domain to open up. Consequently, elimination of this hydrogen bond may decrease the rate of this step. If the rate decreases to be slower than the first conformational change then the V_{max}/K_m will decrease.

In phosphodiester bond formation, the hydrogen bond between Arg841 and the 3-position of guanine is not directly involved. However, this interaction may play a role in accelerating the reaction even though it is not in the catalytic site. The hydrogen bond may position the DNA and consequently the dNTP to be in optimal position for reaction to occur. If an enzyme binds to the transition state more tightly than the substrate, the rate of the reaction will be increased (*36*). Thus, the energy of the hydrogen bond between Arg841 and the 3-position of guanine would not increase the affinity of the substrate but would be used to accelerate the reaction.

In spite of the decreased rates of incorporation of C opposite 3DG, dCTP still is the best base pair partner for 3DG. The V_{max}/K_m for the incorporation of dCTP opposite 3DG is 260-fold larger than that for dTTP. The interaction between the 3-position of guanine and the Klenow fragment is important to catalysis and fidelity. But since removal of this hydrogen bond does not eliminate the preference of dCTP, there must be other interactions which also contribute to the preference of G for dCTP.

$$E + DNA_n \rightleftharpoons E\text{-}DNA_n \qquad \text{DNA binds to polymerase}$$

$$E\text{-}DNA_n + NTP \rightleftharpoons E\text{-}DNA_n\text{-}dNTP \qquad \text{dNTP binds to polymerase}$$

$$E\text{-}DNA_n\text{-}dNTP \rightleftharpoons [E\text{-}DNA_n\text{-}dNTP]^* \qquad \text{conformational change}$$

$$[E\text{-}DNA_n\text{-}dNTP]^* \rightleftharpoons [E\text{-}DNA_{n+1}\text{-}PP_i]^* \qquad \text{phosphodiester bond formation}$$

$$[E\text{-}DNA_{n+1}\text{-}PP_i]^* \rightleftharpoons E\text{-}DNA_{n+1}\text{-}PP_i \qquad \text{relaxation of conformation}$$

$$E\text{-}DNA_{n+1}\text{-}PP_i \rightleftharpoons E\text{-}DNA_{n+1} \qquad \text{release of pyrophosphate}$$

FIGURE 3: Kinetic scheme for the incorporation of a single nucleotide by the Klenow fragment (34).

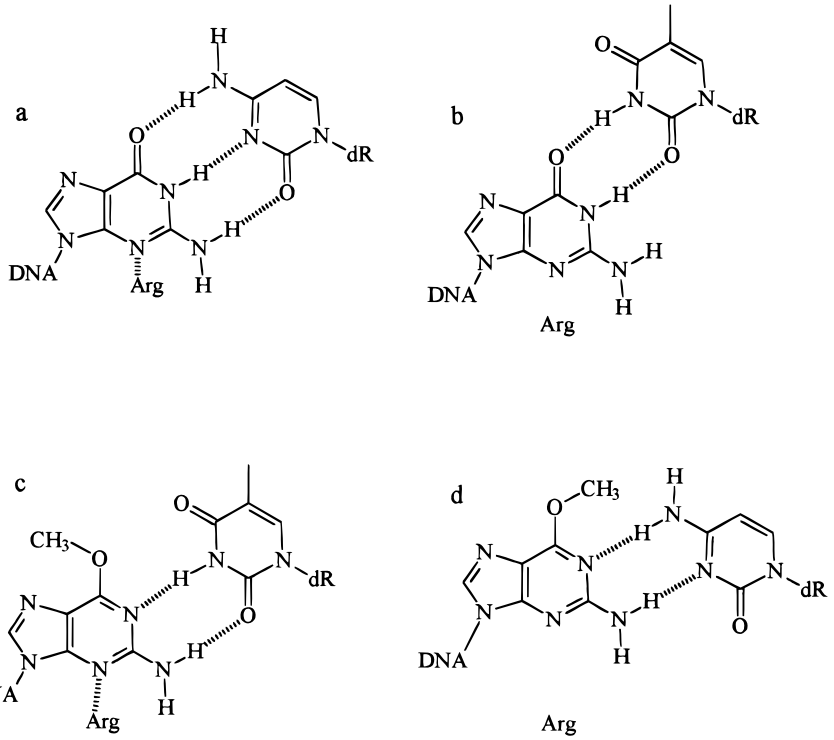

FIGURE 4: Proposed hydrogen bonding schemes during replication: a, G−dCTP; b, G−dTTP; c, O^6mG−dTTP; and O^6mG−dCTP.

The V_{max}/K_m for mispair formation is decreased less than 5-fold when 3DG is substituted for G. This reduction in rate is much less than the 170-fold decrease in the incorporation of dCTP. This result suggests that either the hydrogen bond between Arg841 and the 3-position of guanine does not form during mispair incorporation or the hydrogen bond is not involved in the rate-determining step.

The hydrogen bonding configurations of guanine mispairs (GA, GT, and GG) are distorted from Watson−Crick geometry (*5*, *37*−*39*). The altered geometry may prevent the hydrogen bond between Arg841 and the 3-position of guanine from forming. If it does not form then we would not expect to see a decrease in V_{max}/K_m when G is replaced by 3DG.

Alternatively, there may be a hydrogen bond between Arg841 and the 3-position of guanine during the incorporation of A, T, or G, but we did not detect a decrease in V_{max}/K_m because the effect is in a step which is not rate limiting. The rate-determining step in the generation of mispairs is phosphodiester bond formation (*34*). If the hydrogen bond between Arg841 and the 3-position of guanine is involved in the conformational change, then we may not observe a decreased V_{max}/K_m. If the rate of the conformational change is decreased, we would not observe a decrease in V_{max}/K_m unless the rate of conformational change becomes less than the rate of phosphodiester bond formation.

The methylation of the 6-position of guanine is mutagenic, as thymine is incorporated opposite it more often than cytosine. The preference for the incorporation of thymine may be because it can form a more Watson−Crick-like structure in the active site of the polymerase with O^6mG than does cytosine (*18*). The ability of Kf (exo$^-$) to form a hydrogen bond between Arg841 and the 3-position of O^6-mG when dTTP is bound but not when dCTP is bound may also contribute to the preference of O^6mG to incorporate thymine. To investigate the potential hydrogen bond between Kf (exo$^-$) and the 3-position of O^6mG, we compared the steady-state kinetics of O^6mG and O^6m3DG.

The rate of incorporation of thymine opposite O^6mG was reduced 14 000-fold when the nitrogen at the 3-position of

O^6mG was replaced by carbon. This is a much greater effect than that observed for the incorporation of cytosine opposite guanine and 3DG (170-fold). As in the case for the incorporation of cytosine opposite 3DG, the large decrease in the rate of incorporation opposite O^6m1DG suggests that a hydrogen bond occurs between the 3-position of O^6mG and the polymerase during the incorporation of thymine.

The rate-determining step for the incorporation of dCTP and dTTP opposite O^6mG is the relaxation of the conformation after phosphodiester bond formation (*35*). The preferential incorporation of T occurs due to an larger rate of phosphodiester formation with dTTP than with dCTP as cosubstrate (*35*). From steady-state kinetics we cannot determine which step is affected by the change in substrate. The rate-limiting relaxation of conformation may be decreased, or another step may be affected to a greater extent such that it becomes rate limiting.

However, comparison of the 14 000-fold rate reduction observed with O^6mG−dTTP versus the 170-fold rate reduction observed with G−dCTP may indicate that the hydrogen bond between Arg841 and the 3-position of guanine or O^6-mG is important in the relaxation of conformation. If the substitution of the 3-position of G and O^6mG both decrease the relaxation of conformation to a similar extent, then the O^6mG reaction would exhibit a larger decrease in V_{max}/K_m. With O^6mG−dTTP, a 14 000-fold decrease in this step would directly manifest itself in the V_{max}/K_m. With G−dCTP, only the decrease in rate after the step becomes rate limiting would become apparent in the V_{max}/K_m.

The results with dCTP are more complicated than with dTTP. In the incorporation of cytosine opposite O^6mG, the V_{max}/K_m increased 6-fold upon substitution of the 3-position. This small change in V_{max}/K_m would suggest that the 3-position of O^6mG was not involved in a hydrogen bond during replication. However, the lack of a change in V_{max}/K_m was the result of a 500-fold decrease in V_{max} offset by a 2900-fold decrease in K_m. Perhaps, an intermediate is stabilized such that the K_m is lowered and the catalytic steps are slower.

In summary, the reduction in V_{max}/K_m due to the incorporation of dCTP opposite 3DG with respect to G suggests that a hydrogen bond exists between the Klenow fragment and the 3-position of guanine as illustrated in Figure 4a. This interaction may be important in the ability of the polymerase to discriminate between Watson−Crick base pairs and mismatches (Figure 4b). The relative kinetic parameters also suggest that this interaction exists in the incorporation of dTTP (Figure 4c) and not dCTP opposite O^6mG (Figure 4d) and may be a basis for the mutagenicity of O^6mG.

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