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Identification of Hydrogen Bonds between *Escherichia coli* DNA Polymerase I (Klenow fragment) and the Minor Groove of DNA by Amino Acid Substitution of the Polymerase and Atomic Substitution of the DNA†

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ABSTRACT: DNA polymerases replicate DNA with high fidelity despite the small differences in energy between correct and incorrect base pairs. X-ray crystallographic and structure−activity kinetic experiments have implicated interactions with the minor groove of the DNA as being crucial for catalysis and fidelity. The current hypothesis is that polymerases check the geometry of the base pairs through hydrogen bonds and steric interactions with the minor groove of the DNA. The mechanisms by which these interactions are related to catalysis and fidelity are not known. In this manuscript, we have studied these interactions using a combination of site-specific mutagenesis of *Escherichia coli* DNA polymerase I (Klenow fragment) and atomic substitution of the DNA. Crystal structures have predicted hydrogen bonds from Arg668 to the terminal base on the primer (P1) and Gln849 to its base pair partner (T1). Kinetic studies, however, have implicated the minor groove of the primer terminus but not its base pair partner as being important to catalysis and fidelity. Hydrogen bonds between Arg668 and Gln849 to the DNA were probed with the site specific mutants, R668A and Q849A. Hydrogen bonds from the DNA were probed with three oligodeoxynucleotides which have a guanine or 3-deazaguanine (3DG) at P1, T1, or T2. We found that the pre-steady-state parameter k_{pol} was decreased with R668A (40-fold) and Q849A (150-fold) or with 3DG at P1 (300-fold) or T2 (25-fold). When R668A was combined with 3DG at P1 the decrease in rate was only 80-fold, consistent with a hydrogen bond between Arg668 and P1. In contrast, when the 3DG substitution at P1 was combined with Q849A the rate reduction was 15000-fold. Similar reactions between R668A or Q849A and T2 showed that there are interactions between these sites although the interactions are not as strong as between P1 and R668.

The proficiency of DNA polymerases to incorporate the correct dNTP into DNA is a major factor in the high fidelity of DNA replication. The similarity in energy between correctly and incorrectly paired bases leads to the conclusion that the polymerases must play an active role in the fidelity of DNA replication (*1*−*3*). Interactions between the polymerase and DNA can enhance the fidelity of replication by (1) excluding water from the active site to amplify the energy difference between correct and incorrect base pairs (*4*), (2) restricting the movement of the DNA, thereby increasing the $\Delta G°$ between the correct and incorrect base pairs (*1*), and (3) selecting for the Watson−Crick geometry (*2*). These three mechanisms are not mutually exclusive. By providing a

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check for Watson−Crick geometry, the polymerase would have to restrict the entropy of the DNA and thereby increase the $\Delta G°$ between correct and incorrect base pairs. The geometric selection would probably exclude H_2O from contact with the DNA, thereby increasing $\Delta H°$.

The similar topographies of the Watson−Crick base pairs may allow polymerases to select for correct base pairing based on structure (*5*). The minor groove of the DNA may be crucial for the geometric selection because the position of the two hydrogen bond acceptors (*N*3 of purines and *O*2 of pyrimidines) are similar in Watson−Crick base pairs but different in mispairs (*6*, *7*). Crystal structures of DNA polymerases bound to DNA have shown that most of the interactions occur on the phosphate backbone but there are many interactions between the protein and the minor groove of DNA (*8*−*13*).

The Klenow fragment of *Escherichia coli* DNA polymerase I (KF)[1] is a member of the pol I family of polymerases and has been extensively studied. The crystal structures of this polymerase do not give insight into the mechanism of polymerization because the DNA has been found bound to the exonuclease site (*14*). However, crystal structures of DNA polymerases I of bacteriophage T7 (*10*), *Thermus aquaticus* (*12*), and *Bacillus stearthermophilis* (*8*) are believed to be good models of the catalytically active conformation of KF. From these structures, it would be predicted that Arg668 forms a hydrogen bond with the minor groove of the primer terminus and Gln849 to the minor groove of its base pair partner on the template. Kinetic studies with mutant proteins have implicated both Arg668 and Gln849 as being important for catalysis and fidelity of DNA replication (*15*−*17*).

Studies with nucleotide analogues have indicated that minor groove of DNA plays an important role in the catalysis and fidelity of DNA replication. The adenine analogues 9-methyl-1H-imidazo[(4,5)-b]pyridine (Q) and 4-methylbenzimidazole (Z) were used to show that minor groove hydrogen bonds are needed at the primer terminus but not at the dNTP, T0 or T1 (*18*−*20*). We found similar results with 3-deazaguanine (3DG) in which the G to 3DG substitution at P1 and T2 caused a 300-fold and 40-fold decrease in k_{pol}, respectively. Substitution at other positions from P0 to P5 and T0 to T5 caused less than a 5-fold decrease in k_{pol} (*21*). However, with a different DNA sequence 3DG inhibited KF^- replication when placed at the template base (T0), suggesting that the minor groove interactions may be sequence specific (*22*). 3-Deaza-dATP also inhibits KF, although whether the insertion or extension step was inhibited is not known (*23*). Different results were observed with dCTP and dTTP analogues lacking the minor groove carbonyl groups (*24*). Lack of insertion implicates minor groove binding to the incoming dCTP and dTTP in contrast to dGTP (*21*) and dATP (*19*).

Several studies support the importance of a hydrogen bond acceptor in the minor groove of the primer terminus (P1). In contrast, the importance of a hydrogen bond acceptor at T1 is not supported by the kinetic studies. In this manuscript, we further examined the interactions between the minor groove of the DNA and the KF^- using a combination of atomic substitution of the DNA and site specific mutations of the protein. In this way, we have additional evidence that the minor groove of the primer terminus forms a hydrogen bond with Arg668.

EXPERIMENTAL PROCEDURES

General. [^{33}P]ATP was purchased from Amersham at 6000 Ci/mmol. T_4 polynucleotide kinase, and wild-type *E. coli* DNA polymerase I (Klenow fragment) with the proofreading exonuclease inactivated (KF^-) were obtained from Promega. The double mutants R668A D424A and Q849A D424A were a gift of Catherine Joyce of Yale University. The dNTPs (ultrapure grade) were purchased from Pharmacia, and the concentrations were determined by UV absorbance (*25*). The oligonucleotides containing 3DG were synthesized, purified by PAGE followed by reverse-phase HPLC, and characterized by enzymatic hydrolysis with HPLC analysis (*22*, *26*, *27*). The concentrations of oligodeoxynucleotides were determined from the absorbance at 260 nm, using the method of Borer (*28*) in which it was assumed that the spectral properties of 3DG were identical to G. The primer was ^{33}P-labeled with γ-[^{33}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 (*27*).

Pre-Steady-State Kinetics. The reaction was initiated by the addition of 15.9 μL of dNTP in water to 16.4 μL of DNA−enzyme solution at 25 °C with a KinTek-3 rapid quench instrument. The composition of the buffer during the reaction was 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM DTT, 100 μg/mL BSA. The concentration of the DNA was 75 nM and the polymerase was 100 nM. The concentration of dNTPs varied from 0 to 500 μM. The reactions were quenched by the addition of 300 mM EDTA.

Product Analysis. 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, and 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 visualized with a Bio-Rad GS 250 Molecular Imager. The progress of the reaction was quantitated by dividing the total radioactivity in the product band by the radioactivity in the product and reactant bands.

Data Analysis. Data were fitted by nonlinear regression using the program GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). Data from the pre-steady-state reactions were fitted to eq 1

$$Y = A\,(1 - e^{-kt}) \qquad (1)$$

in which Y is the product formed, A the total amount of DNA reacted, and k is the pseudo-first-order rate for the dNTP incorporation. The k values for these experiments were fitted to eq 2, where k_{pol} is the maximum rate of dNTP incorpora-

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

[1] Abbreviations: 3DG, 3-deazaguanine; BSA, bovine serum albumin; dNTP, 2′-deoxyribose nucleotide 5′-*O*-triphosphate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; G, guanine; KF, Klenow fragment of *E. coli* DNA polymerase I; KF^-, Klenow fragment of *E. coli* DNA polymerase I with the proofreading exonuclease inactivated; PAGE, polyacrylamide gel electrophoresis.

Scheme 1: Oligodeoxynucleotide Duplexes Used to Examine Hydrogen Bonds between KF$^-$ and the Minor Groove of DNA[a]

	Sequence
P1(X)	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 C G T C A G C G T C -5'
T1(X)	5'- G C A C C G C A G A C G C A G C -3'
	3'- C G T G G C G T C T G C G T C **X** T C A G C G T C -5'
T2(X)	5'- G C A C C G C A G A C G C A G C A -3'
	3'- C G T G G C G T C T G C G T C **X** T C A G C G T C -5'

[a] X = G or 3DG.

tion and K_d^{dNTP} is the equilibrium dissociation constant for the interaction of dNTP with the polymerase−DNA complex.

RESULTS

In this manuscript, we have used atomic substitution on the DNA and amino acid substitution of the protein to investigate whether we can identify both ends of the hydrogen bonds between KF$^-$ and DNA. Oligodeoxynucleotides **P1**, **T1**, and **T2** (Scheme 1) were used as a probe for the importance of the minor groove of the terminal base pair and the template nucleotide of the penultimate base pair. KF$^-$, R668A, and Q849A were employed to determine the functions of Arg668 and Gln849.

Pre-steady-state kinetic analyses were performed with enzyme in excess. The reactions went to completion and pseudo-first-order rate constants (k) were obtained. The k_{pol} and K_d values, obtained by plotting k versus [dNTP], are presented in Table 1. The incorporation of a single nucleotide onto **P1(G)**, **T1(G)**, and **T2(G)** proceeded with similar k_{pol} and K_d values. The k_{pol} ranged from 140 to 590 s^{-1} and the K_d varied from 10 to 30 μM. When the mutant enzymes were used, decreases in rate were observed. With R668A, the k_{pol} decreased 40-, 350-, and 100-fold with **P1(G)**, **T1(G)**, and **T2(G)**, respectively. With Q849A, the k_{pol} decreased 140-, 6-, and 50-fold with **P1(G)**, **T1(G)**, and **T2(G)**, respectively. These values are similar to previously reported results (*16*, *17*). When **P1(3DG)**, **T1(3DG)**, and **T2(3DG)** were used as substrates, the changes in k_{pol} values depended on the combination of which substrate and which enzyme were involved in the reaction. A summary of the results combining the modifications in the DNA and protein is shown in Figure 1.

Figure 1 illustrates the changes in k_{pol} associated with changes in substrate or polymerase. Panel A represents reactions with **P1**, panel B with **T1**, and panel C with **T2**. In each panel, the rates are normalized to the reaction of wild-type KF$^-$ with G in the oligodeoxynucleotide. As illustrated in Figure 1A, the G to 3DG substitution in P1 produced a decrease in k_{pol} over 2 orders of magnitude with KF$^-$. Large decreases were observed when **P1(G)** was reacted with R668A (40-fold decrease) and Q849A (140-fold decrease). The reactivities of R668A and Q849A were also diminished due to 10-fold increases in K_d. When the G to 3DG substitution was combined with the change in protein, an additional 100-fold decrease in rate was observed with Q849A. However the reaction between **P1(3DG)** and R668A was not any slower than that between **P1(G)** and R668A or **P1(3DG)** and KF$^-$. These results are consistent with an interaction between Arg668 and the minor groove of P1.

Panel B shows the changes in rate associated with the substitution of G to 3DG at **T1**. As was observed previously, substitution of the hydrogen bond acceptor on the DNA reduced the k_{pol} to a small extent (only 3-fold) with KF$^-$ (*19*, *21*). In the amino acid substitutions, the replacement of Arg668 decreased the k_{pol} to a much greater extent than the replacement of Gln849 (350- to 6-fold, respectively). When the G to 3DG substitution was combined with the amino acid substitution, only small (5−fold) additional decreases were observed. Thus, changing either Arg668 or Gln849 to Ala did not change the protein such that an interaction to the minor groove at T1 became important.

At the T2 position, the G to 3DG substitution decreased the k_{pol} 25-fold with KF$^-$. The reduction in k_{pol} was 10-fold less than that observed at the primer terminus due to the G to 3DG substitution. The reaction between **T2(G)** and R668A and Q849A were decreased 100- and 50-fold, respectively, compared with KF$^-$. With the mutant proteins, when **T2(3DG)** replaced **T2(G)** the k_{pol} decreased only 3-fold for R668A and 2-fold for Q849A. These decreases are 10-fold less than the 25-fold decrease observed with wild-type KF$^-$. This result suggests that alteration of either Arg668 or Gln849 results in a protein in which a hydrogen bond to the minor groove of T2 does not enhance catalysis, and suggest an interconnection between Arg668 and Gln849 with T2.

DISCUSSION

DNA replication is a complex process, consisting of at least seven individual steps (*29*, *30*). The correct nucleotide is incorporated because the activation energies for several of the reactions increase during the incorporation of an incorrect dNTP (*31*, *32*). To determine the mechanisms by which polymerases replicate DNA with high fidelity, the structures of the transition states of these reactions must be investigated. X-ray crystallography experiments provide the structure of substrate-enzyme complexes with high resolution. From X-ray crystallographic studies of *Bacillus*, *Taq*, and T7 DNA polymerases (*8*, *10*, *12*), it would be predicted that Arg668 and Gln849 of KF$^-$ form hydrogen bonds with the terminal base pair adjacent to the newly forming base pair. Arg668 would be predicted to bind to the minor groove of the primer terminus (P1) and Gln849 to the base pair partner on the template (T2). These structures, however, are equilibrium structures of ground-state intermediates. These structures, however, are important in that they are an excellent starting point from which predictions can be made about the structures of the transition states.

Kinetic studies can be used to test the proposed transition state structures through structure−activity relationships. Amino acid substitution of Arg668 to Ala and Gln849 to Ala have produced polymerases with significantly reduced catalytic activities (*16*, *17*). The R668A polymerase also has reduced fidelity of incorporation (*33*). These findings are consistent with the hypothesis that Arg668 and Gln849 make hydrogen bonds to the minor groove of the terminal base pair. However, amino acid substitution changes more than just a single hydrogen bond. The loss of the arginine and glutamine side chains could result in decreased activity through a variety of mechanisms.

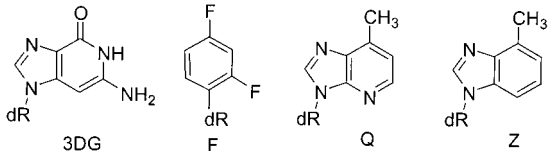
The potential hydrogen bond between R668A and Gln849 and the minor groove of the terminal base pair has been

Table 1: Pre-Steady-State Kinetic Parameters for the Incorporation of dNTP Opposite N with Either G or 3DG in the DNA[a]

			dNTP							
			5'- P5	P4	P3	P2	P1	↓		
			3'- T5	T4	T3	T2	T1	T0	T-1	T-2 -5'
			G[b]				3DG[b]			
pol	DNA	dNTP/N[c]	k_{pol} (s^{-1})		K_d (μM)		k_{pol} (s^{-1})		K_d (μM)	
KF⁻	**P1**	dCTP/G	140 ± 30		30 ± 15		0.43 ± 0.12		20 ± 10	
R668A	**P1**	dCTP/G	3.5 ± 0.9		190 ± 110		1.8 ± 0.6		140 ± 70	
Q849A	**P1**	dCTP/G	1.0 ± 0.2		230 ± 90		0.0095 ± 0.0018		230 ± 70	
KF⁻	**T1**	dATP/T	590 ± 140		10 ± 2		200 ± 20		10 ± 1	
R668A	**T1**	dATP/T	1.7 ± 0.2		350 ± 60		0.35 ± 0.01		180 ± 40	
Q849A	**T1**	dATP/T	91 ± 8		320 ± 110		20 ± 4		130 ± 40	
KF⁻	**T2**	dGTP/C	440 ± 20		8 ± 2		25 ± 2		3 ± 1	
R668A	**T2**	dGTP/C	4.2 ± 0.5		150 ± 50		1.5 ± 0.2		200 ± 90	
Q849A	**T2**	dGTP/C	8 ± 1		180 ± 80		4.2 ± 0.5		230 ± 90	

[a] Reaction was carried our in 50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM DTT, 100 μg/mL BSA at 25 °C. The polymerase concentration was 100 nM and the DNA concentration was 75 nM, while the dNTP concentration was varied up to 1 mM. [b] Identity of the variable base in the DNA [c] Identity of the incoming dNTP and the template base (T0).

examined with purine analogues in which the nitrogen at the 3-position has been changed to carbon. The catalytic activity of KF with oligodeoxynucleotides in which the terminal base pair (T1/P1) was Q/F or F/Q was 100-fold less than A/T or T/A base pair (*18*, *20*). The V_{max}/K_m dropped an additional two-orders of magnitude when Z replaced Q at P1 but not at T1. The Q/F base pair differs from the A/T base pair such that a decrease in rate is observed. Nevertheless, the Q/F base pair is similar enough to A/T, such that the minor groove interactions are intact. These results were corroborated with the more conservative substitution of G to 3DG, in which 3DG differs from G at only one position (*21*). These results are consistent with a critical hydrogen bond between Arg668 and the N3-position of the purine at P1. The lack of an effect at T1 indicates that the hydrogen bond between Gln849 and purines at T1 is not essential to the rate-limiting step of the reaction.



Kinetic experiments have shown that both Arg668 and the N3 position of a purine at the primer terminus are involved in interactions critical to DNA replication. The hypothesis that they form a hydrogen bond is based upon the X-ray crystallography data from *Taq*, T7, and *Bacillus* DNA polymerase I, in which the distance between the terminal nitrogen on arginine and the minor groove hydrogen bond acceptors are 2.6, 2.8, and 2.8 Å, respectively (*8*, *10*, *12*). However, the structures may change during the transition states. In this manuscript, the interaction between Arg668 and the primer terminus was examined by kinetic experiments by the reaction of KF^- mutants with DNA containing 3DG. If the loss of the hydrogen bond between Arg668 and P1 is the cause of the decrease in rate of R668A and DNA with 3DG at P1, then the double substitution should not be less reactive than either single substitution. This rationale is illustrated in Figure 2. Figure 2a shows the hydrogen bonding interaction between Arg668 and the 3-position of G at the primer terminus. Figure 2b illustrates that the reaction between R668A and DNA containing G would lack this hydrogen bond. Similarly, the reaction between wild-type KF^- and DNA containing 3DG would also lack this hydrogen bond (Figure 2c). Figure 2d illustrates the interaction between R668A and 3DG in which the double substitution results in the loss of only a single hydrogen bond between the DNA and polymerase. If the decrease in rate caused by the individual changes are due to this hydrogen bond, then changing both ends of the hydrogen bond would not decrease the rate further.

Experimentally, it was found that changing either Arg668 or the N3 of guanine produced complexes that are inefficient at catalyzing DNA replication. The Arg668 to Ala substitution reduced k_{pol} 40-fold, while the G to 3DG substitution at P1 reduced k_{pol} 300-fold. When the modifications are combined, no additional decrease in k_{pol} was observed. This

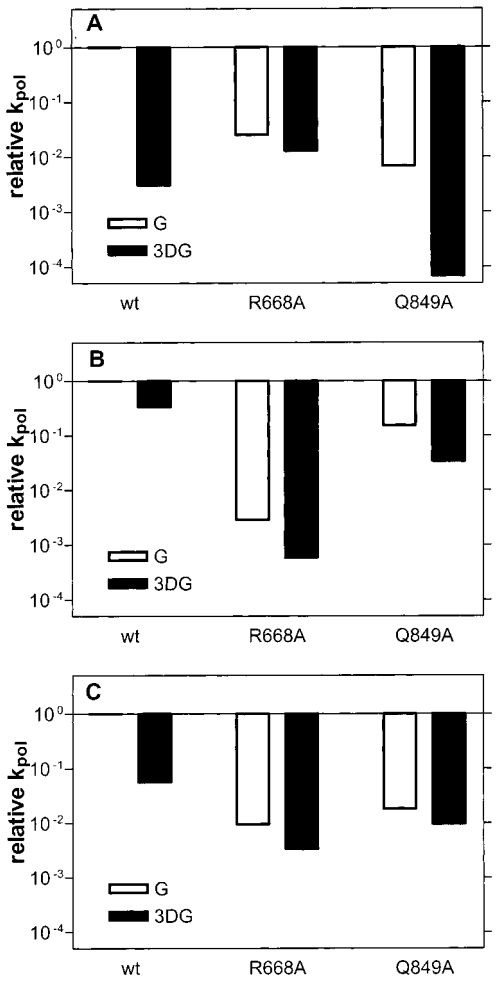


FIGURE 1: Changes in k_{pol} due to amino acid substitution of KF$^-$ and atomic substitution of the DNA. The values are normalized to wild-type KF$^-$ and G in the DNA. The open bars represent the DNA containing G while the solid bars represent the DNA containing 3DG. The reactions with **P1** are shown in panel A, **T1** in B, and **T2** in C.

result indicates that the Arg668 to Ala substitution and the G to 3DG substitution decrease k_{pol} by mechanisms that are the same or are interdependent. It is consistent with a hydrogen bond between Arg668 and P1.

Drastically different results were observed with Q849 and the substitutions at P1. The Gln849 to Ala substitution reduced k_{pol} 140-fold, while the k_{pol} of the reaction between Q849A and **P1(3DG)** was 15000-fold less than with wild-type KF$^-$ and **P1(G)**. The reduction in rate between Q849A and P1(3DG) is consistent with the amino acid and nucleotide substitutions acting independently. If the 300-fold rate reduction due to the G to 3DG substitution were combined with the 140-fold rate reduction of the amino acid substitution, the predicted rate reduction would be 42000-fold. The 15000-fold decrease in k_{pol} is only 3-fold less than the predicted value if the substitutions act to decrease the k_{pol} by independent mechanisms.

The interaction between the polymerase and the minor groove of T2 was also analyzed. The G to 3DG substitution at T2 reduced the k_{pol} 25-fold and did not affect the K_d. The 25-fold reduction in k_{pol} is an order of magnitude less than that observed at P1. Clearly, a hydrogen bond to T2 is not as important as the one to P1. Perhaps the protein is bound to this position via a water molecule. Surprisingly, the k_{pol}

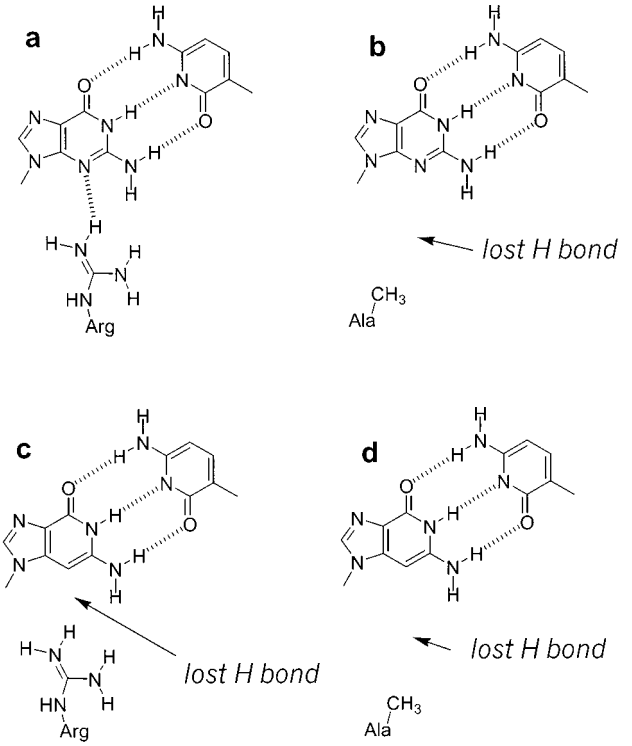


FIGURE 2: Potential interactions between G at the primer terminus and residue 668 of KF$^-$ with the following polymerases and substrates: (a) KF$^-$ and **P1(G)**, R668A and **P1(G)**, (c) KF$^-$ and **P1(3DG)**, and (d) R668A and **P1(3DG)**.

for the R668A and Q849A catalyzed reactions were not significantly affected by the G to 3DG substitutions. This result suggests that both Arg668 and Q849 are involved with the hydrogen bond to the minor groove of T2. Perhaps both Arg668 and Gln849 are involved with the hydrogen bond network between the minor groove of the DNA and water molecules.

In summary, a functional connection has been shown between Arg668 of KF$^-$ and the minor groove of the primer terminus using amino acid substitution of the polymerase and atomic substitution of the DNA. These results are consistent with a hydrogen bond between these two sites during the rate-limiting step of DNA replication. The role of this hydrogen bond may be to orient Arg668 to enhance phosphodiester bond formation. Crystallography structures show that while one imino group of the critical Arg can from a hydrogen bond with the minor groove of the P1 position, the other imino group is directed toward the ring oxygen of the incoming dNTP (*8*, *10*, *12*). Perhaps when Arg668 is correctly positioned by forming a hydrogen bond with the primer terminus, it can also catalyze phosphodiester bond formation by pulling the incoming dNTP toward the nucleophilic 3′-hydroxyl group of the primer strand.

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