Escherichia coli DNA Polymerase I (Klenow Fragment) Uses a Hydrogen-bonding Fork from Arg<sup>668</sup> to the Primer Terminus and Incoming Deoxynucleotide Triphosphate to Catalyze DNA Replication*<sup>§</sup>

Received for publication, May 24, 2004
Published, JBC Papers in Press, June 20, 2004,
DOI 10.1074/jbc.C400232200

Aviva S. Meyer, Maureen Blandino, and Thomas E. Spratt‡

From the Institute for Cancer Prevention, American Health Foundation Cancer Center, Valhalla, New York 10595

Interactions between the minor groove of the DNA and DNA polymerases appear to play a major role in the catalysis and fidelity of DNA replication. In particular, Arg<sup>668</sup> of <i>Escherichia coli</i> DNA polymerase I (Klenow fragment) makes a critical contact with the N-3-position of guanine at the primer terminus. We investigated the interaction between Arg<sup>668</sup> and the ring oxygen of the incoming deoxynucleotide triphosphate (dNTP) using a combination of site-specific mutagenesis of the protein and atomic substitution of the DNA and dNTP. Hydrogen bonds from Arg<sup>668</sup> were probed with the site-specific mutant R668A. Hydrogen bonds from the DNA were probed with oligodeoxynucleotides containing either guanine or 3-deazaguanine (3DG). Hydrogen bonds from the incoming dNTP were probed with (1'R,3'R,4'R)-1-[3-hydroxy-4-(triphosphorylmethyl)cyclopent-1-yl]uracil (dcUTP), an analog of dUTP in which the ring oxygen of the deoxyribose moiety was replaced by a methylene group. We found that the pre-steady-state parameter <i>k</i><sub>pol</sub> was decreased 1,600 to 2,000-fold with each of the single substitutions. When the substitutions were combined, there was no additional decrease (R668A and 3DG), a 5-fold decrease (3DG and dcUTP), and a 50-fold decrease (R668A and dcUTP) in <i>k</i><sub>pol</sub>. These results are consistent with a hydrogen-bonding fork from Arg<sup>668</sup> to the primer terminus and incoming dNTP. These interactions may play an important role in fidelity as well as catalysis of DNA replication.

The high fidelity of DNA replication synthesis is accomplished despite the similarity in energy between correctly and incorrectly paired bases (1, 2). Since inter-strand hydrogen bonds cannot account for this selectivity (3, 4), other mechanisms have been proposed to supply the fidelity (reviewed in Ref. 5). These include solvation (6), base stacking (7, 8), steric exclusion (3, 4, 9), and minor groove binding (10). These mechanisms are not mutually exclusive and may all enhance selection for the correct base pairs.

The minor groove has been suggested to be a site by which polymerases can check geometry because the O<sub>2</sub>-position of pyrimidines and the N-3-position of purines occupy similar spatial orientations as well as being hydrogen bond acceptors (10). Crystal structures of DNA polymerases bound to DNA have shown that there are many interactions between the protein and the minor groove of DNA (11–15). Site-directed mutagenesis studies have implicated several amino acid residues as being important for catalysis and fidelity of DNA replication (15–20). Similarly, the use of purine analogs such as 3-deazaadenine (21, 22), 3-deazaguanine (3DG) (23, 24), 9-methyl-1H-imidazolo[4,5-b]pyridine and 4-methylbenzimidazole (25, 26), and the pyrimidine analogs difluorotoluene (25), 2-aminoypyridine, and 3-methyl-2-pyrindone (27) also have implicated the minor groove of the DNA as being crucial.

X-ray crystallographic studies of polymerases that have a structure similar to that of KF<sup>·</sup> (Taq) (13) and T7 (14) DNA polymerases predict a hydrogen-bonding interaction between an arginine residue (Arg<sup>668</sup> of KF<sup>·</sup>) and the N-3-position of a purine or the O<sub>2</sub>-position of a pyrimidine at the primer terminus. Site-specific mutagenesis of Arg<sup>668</sup> implicated the importance of this residue in the catalysis (16, 28) and fidelity of DNA replication (20). Nucleotide analog studies have indicated that the N-3-position of a purine at the primer terminus is essential to catalysis (24, 26) and fidelity of DNA replication.

The question that we address in this manuscript is how the interaction at the primer terminus influences the rate of incorporation of the incoming dNTP. The ternary crystal structures of Taq (13) and T7 (14) DNA polymerases bound to DNA and an incoming dNTP show that while one imino group of the critical arginine residue is in hydrogen bonding distance with the O<sub>2</sub>-cytosine (Taq polymerase) or N-3 adenine (T7 polymerase) with primer terminus, the other imino group may potentially form a hydrogen bond with the ring oxygen of the incoming dNTP. This interaction may be the mechanism by which disruption of the arginine-primer terminus hydrogen bond decreases the rate of polymerization. To examine this hypothesis we compared the kinetic parameters of the reactions containing wild-type <i>Escherichia coli</i> DNA polymerase I (Klenow fragment) with the exocyclic enoneucleic activity inactivated (KF<sup>·</sup>) or the R668A mutant, DNA containing guanine or 3-deazaguanine at the primer terminus, and dUTP or its carbocyclic analog (1'R,3'R,4'R)-1-[3-hydroxy-4-(triphosphorylmethyl)cyclo-

---

* This work was supported by National Institutes of Health Grant CA 75074. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, Fig. S1, and Refs. 1–10.

‡ To whom correspondence should be addressed. Tel.: 914-789-7289; Fax: 914-729-3344; E-mail: tspratt@fcp.us.

This paper is available on line at http://www.jbc.org
Experimental Procedures

General—[^32P]ATP was purchased from Amersham Biosciences at 6,000 Ci/mmol. T4 polynucleotide kinase and the Klenow fragment of E. coli DNA polymerase I with the exonuclease activity inactivated (KF) were obtained from United States Biochemical. The double mutant R668A and D424A KF was a gift from Catherine Joyce of Yale University. The D424A mutation knocks out the proofreading exonuclease activity. The dNTPs (ultrapure grade) were purchased from Amersham Biosciences, and the concentrations were determined by UV absorption (28). dcUTP was synthesized as described in the supplemental literature (see supplemental material).

The oligonucleotides containing 3DG were synthesized, purified by PAGE followed by reverse-phase HPLC, and characterized by enzymykinetic hydrolysis with HPLC analysis (23, 24). The concentrations of oligodeoxynucleotides were determined from the absorbance at 260 nm, using the method of Borer (30) in which it was assumed that the spectroscopic properties of 3DG were identical to G. The primer was ^[^32P]P-labeled with [γ-[^32P]]ATP and annealed with a 50% excess of the template as described previously (24).

Pre-steady-state Kinetics—Rapid reactions were initiated by the addition of 15.9 μl of dNTP and MgCl2 in water to 16.4 μl of DNA-enzyme solution at 25 °C with a KinTek-3 rapid quench instrument. Slow reactions were carried out by hand. The composition of the buffer during the reaction was 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 5 mM DTT, 100 μg/ml BSA. Typically the DNA concentration was 20 nm, and the polymerase concentration was 50 nm. The concentration of dNTPs varied from 10 to 1,000 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 20% acrylamide (19:1, acrylamide:N,N'-methylene bisacrylamide), 7% urea in 1× TBE buffer (0.089 × Tris, 0.069 × boric acid, 0.002 × NaOH, EDTA). The size of the gel was 40 × 33 × 0.4 cm and was run at 2,500 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(s) by the radioactivity in the product and reactant bands. Multiple product bands appeared when the incorrect dNTP was added to the reaction.

Data Analysis—Data were fitted by nonlinear regression using the program Prism version 4.0 for Windows (GraphPad Software, San Diego, CA (www.graphpad.com)). The time course data were fit to Equation 1, where P is the product formed, A is the total amount of DNA reacted, and k is the first order rate constant for the dNTP incorporation. The kpol and kred values were obtained by fitting k to [dNTP] according to Equation 2.

\[ P = A(1 - e^{-kt}) \]  
\[ k = k_{pol}[dNTP]/[dNTP] + K_s \]  

**RESULTS**

The interactions between Arg668, the minor groove of the primer terminus, and the ring oxygen of the incoming dNTP were evaluated kinetically using R668A, oligodeoxynucleotides with 3DG at the primer terminus, and dcUTP. These substitutions will be effective in examining the proposed interactions only if each modification does not drastically alter the chemistry of the enzyme or nucleotide analog except at the site of modification. Arg668 has been shown to be important to catalysis and fidelity of DNA replication (16, 20, 28, 31), but the overall structure of the protein appears unaffected by the substitution (28). 3DG has been used to examine minor groove interactions and is a good substrate at some sites in the DNA but not others and therefore, is a good mimic of guanine (24, 32). Several nucleotide analogs in which the deoxyribose moiety was replaced with a carbocyclic base have been examined and they inhibit DNA synthesis by competitive inhibition or chain termination depending on the polymerase (33–35). The structure of a carbocyclic analog of dA paired with dT in an oligodeoxynucleotide duplex is similar to that of dA suggesting that dcUTP should be a good mimic of dUTP (36).

The incorporation of dUTP and dcUTP opposite dA was examined by reacting 50 nM KF with 20 nM of the appropriate primer-template. Since [KF] > [dNTP], the formation of product could be fit to a first order equation. The first order rate constants were plotted against the concentration of the incoming dNTP to obtain the kpol and kred values. The kinetic parameters are listed in Table I, and the relative kpol values are presented in Fig. 2.

As was observed previously, the Arg to Ala and G to 3DG substitutions support the existence of a critical hydrogen bond between Arg668 and the minor groove of the primer terminus (24). In the present study, the G to 3DG substitution caused a 2,000-fold decrease in kpol (reaction 1 versus 2 in Table I), while G with the template base the Arg668 to Ala substitution induced a 1,600-fold decrease in kpol (reaction 1 versus 3). The double substitution of G to 3DG and Arg668 to Ala did not cause any additional decrease in kpol from the single modifications (reactions 2 and 3 versus 4). The observation that the effects of the two substitutions were not cumulative indicates that there is a hydrogen bond interaction between Arg668 and the N-3-position of guanine at the primer terminus (24).

In similar experiments, we examined the relationship between Arg668 and the deoxyribose ring oxygen of the incoming dNTP. The replacement of dUTP by dcUTP decreased the kpol ∼1,200-fold with a 4-fold increase in Ks (reaction 1 versus 5). Thus, the ring oxygen is very important to reactivity but only slightly affects the binding of the dNTP. The double substitution of Arg to Ala and dUTP to dcUTP resulted in a 50-fold decrease in kpol from either of the single substitutions (reactions 3 and 5 versus 7). This decrease in rate falls between no effect and the ∼1,500-fold decrease in kpol caused by the single substitutions. This result suggests that there is an interaction between Arg668 and the ring oxygen. However, the lack of this hydrogen bond does not entirely explain the decrease in kpol caused by the dUTP to dcUTP substitution.

We also examined the relationship between the ring oxygen of dUTP and the 3-position of guanine at the primer terminus. As discussed above, the decrease in rates associated with the
substitutions shows similar results and supports a mechanistic approach to enzymatic processes. The incoming nucleotide is dUTP (shown) or dcUTP (reactive group), and the incoming nucleotide is dUTP (shown) or dcUTP (reaction 7). The double substitution decreased the kinetic parameter (Table I). The decrease in the k_{pol} parameter, determined with 20 mM DNA and 50 mM KF, was 10-fold from 20 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 5 mM DTT, 100 μg/ml BSA at 25 °C with 20 mM DNA and 50 mM KF. The concentration of dNTPs varied from 0 to 1,000 μM. The relative K_d versus k_{pol} indicates the importance of these sites (reaction 1). Individual substitution of G with 3DG and dUTP with dcUTP does not indicate a direct interaction between these two positions, the primer terminus of the DNA. Since the crystal structures show that there are interactions between the minor groove of the DNA and the polymerase (11–15). A critical contact between KF and the minor groove of the DNA is made by Arg668 (24, 37). This contact is essential for both the catalytic efficiency (16, 28) and fidelity (29) of the protein and may play a role in the transfer of the DNA between the catalytic and exonuclease sites (22). In this manuscript, we have described experiments indicating that in addition to forming a hydrogen bond with the N-3-position of guanine at the primer terminus it also forms a hydrogen bond with the deoxyribose ring oxygen of the incoming dNTP.

**Table I**

Kinetic parameters for the incorporation of dUTP or dcUTP opposite A with G or 3DG at the primer terminus and with wild-type of R668A KF.

| Reaction | dNTP | Polymerase | X^a | k_{pol} | K_d |
|----------|------|------------|-----|---------|-----|
| 1        | dUTP | KF^-       | G   | 78 ± 7  | 33 ± 9 |
| 2        | dUTP | KF^-       | 3DG | 0.038 ± 0.002 | 57 ± 10 |
| 3        | dUTP | R668A      | G   | 0.048 ± 0.0021 | 17 ± 3 |
| 4        | dUTP | R668A      | 3DG | 0.11 ± 0.01 | 22 ± 3 |
| 5        | dcUTP| KF^-       | G   | 0.045 ± 0.020 | 124 ± 50 |
| 6        | dcUTP| KF^-       | 3DG | 0.099 ± 0.0002 | 32 ± 16 |
| 7        | dcUTP| R668A      | G   | 0.0009 ± 0.0001 | 19 ± 10 |
| 8        | dcUTP| R668A      | 3DG | 0.0008 ± 0.0002 | 21 ± 10 |

^a Identity of nucleotide at primer terminus. See Fig. 2 for sequence of DNA.

**DISCUSSION**

One mechanism by which polymerases can replicate Watson-Crick base pairs is by geometric selection. For this to occur, the polymerases must have contact points with the DNA at positions in which the topology and chemistry is indistinguishable among the four base pairs. The minor groove is thought to be important for base pair independent contacts because the N-3-position of purines and the O2'-position of pyrimidines occupy similar steric positions and have similar chemistry, that of being hydrogen bond acceptors (10). In support of this hypothesis, x-ray crystal structures of several polymerases have shown that there are interactions between the minor groove of the DNA and the polymerase (11–15).

A critical contact between KF and the minor groove of the DNA is made by Arg668 (24, 37). This contact is essential for both the catalytic efficiency (16, 28) and fidelity (29) of the protein and may play a role in the transfer of the DNA between the catalytic and exonuclease sites (22). In this manuscript, we have described experiments indicating that in addition to forming a hydrogen bond with the N-3-position of guanine at the primer terminus it also forms a hydrogen bond with the deoxyribose ring oxygen of the incoming dNTP.

**FIG. 2.** A. Representation of the reaction studied. Amino acid 668 is either Arg (shown) or Ala, the primer terminus is G (shown) or 3DG (DG), and the incoming nucleotide is dUTP (shown) or dcUTP (cU). B, relative k_{pol} determined with 20 mM DNA and 50 mM KF, in 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 5 mM DTT, 100 μg/ml BSA at 25 °C. The k_{pol} is relative to that performed with KF, unmodified DNA, and dUTP. The lower section shows the changes associated with each bar.
hydrogen bond acceptor may be a physical property that the polymerase can check to determine whether a base pair is in the Watson-Crick geometry. Thus, these interactions may explain how the rates of insertion are decreased with mispairs at either the terminal base pair or the newly forming base pair.

Two mechanisms can be envisioned of how Arg\textsuperscript{668} would enhance the catalytic efficiency of correct base pairs but not Watson-Crick base pairs and another in which the residue plays a regulatory role. Arg\textsuperscript{668} could actively be involved in phosphodiester bond formation by aligning the incoming dNTP into correct position for reaction to occur. Its function may be to align the \( \alpha \)-phosphate of the incoming dNTP with the 3'-hydroxyl group of the primer terminus. Alternatively, Arg\textsuperscript{668} may lower the activation energy of phosphodiester bond formation by pulling the dNTP onto the primer terminus. Due to the relative positions of the correct \textit{versus} incorrect base pairs, this process would occur only with Watson-Crick base pairs at the terminal base pair and the newly forming base pair. With a mispair at either position, the arginine fork would help to orient the incoming dNTP into an inactive configuration and thus, decrease the rate of replication.

Arg\textsuperscript{668} may also modulate the activity of the polymerase by an indirect mechanism. The position of Arg\textsuperscript{668} may be dependent on the positions of the primer terminus and incoming dNTP. If they are not in Watson-Crick geometry then the altered Arg\textsuperscript{668} position may disrupt the overall geometry of the catalytic site to inhibit the rate-limiting conformational change and phosphodiester bond formation. Since Arg\textsuperscript{668} is on the interface of the fingers and palm domain its position may be vital to the overall structure of the binding/active site.

The kinetics of \textit{E. coli} DNA polymerase I can be described by seven steps illustrated in Fig. 3 (38–40). The rate-limiting step for correct replication is the formation of the phosphodiester bond (step 3) (40). However, the rate of phosphodiester bond formation slows down to become rate-limiting for mispair formation (38). Thus, the decrease that we observed in \( k_{\text{pol}} \) could be due to either a decrease in the rate-limiting conformational change or phosphodiester bond formation. If Arg\textsuperscript{668} plays an active role, we would speculate that these changes would affect phosphodiester bond formation, while if it is a regulatory role we would speculate that the changes affect the conformational change.
Escherichia coli DNA Polymerase I (Klenow Fragment) Uses a Hydrogen-bonding Fork from Arg to the Primer Terminus and Incoming Deoxynucleotide Triphosphate to Catalyze DNA Replication

Aviva S. Meyer, Maureen Blandino and Thomas E. Spratt

J. Biol. Chem. 2004, 279:33043-33046.
doi: 10.1074/jbc.C400232200 originally published online June 20, 2004

Access the most updated version of this article at doi: 10.1074/jbc.C400232200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/06/25/C400232200.DC1

This article cites 38 references, 14 of which can be accessed free at http://www.jbc.org/content/279/32/33043.full.html#ref-list-1