A Conformational Change in *E. coli* DNA Polymerase I (Klenow Fragment) Is Induced in the Presence of a dNTP Complementary to the Template Base in the Active Site†

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ABSTRACT: It is well established that the insertion of a nucleotide into a growing DNA chain requires a conformational change in the structure of a DNA polymerase. These enzymes have been shown to bind a primer-template in the open conformation and then upon binding of a complementary dNTP undergo a conformational rearrangement to the closed ternary complex. This movement results in the positioning of the incoming nucleotide in the proper geometry for the nucleophilic attack by the 3′-hydroxyl of the primer. In this work, tryptic digestion experiments were performed to detect this conformational change in the structure of the exonuclease-deficient DNA polymerase I (Klenow fragment). Three distinct digestion patterns were observed: one for the polymerase alone, one for the binary complex with the primer-template, and one for the ternary polymerase−DNA−dNTP complex. The latter conformational change leads to a stable ternary closed complex formation only when the correct nucleotide is present in the reaction mixture. Positioning of nucleotides with incorrect geometry in the protein active site inhibits or eliminates formation of the closed complex. Similarly, this conformational change is inhibited when the primer terminus of the DNA molecule is altered by the presence of the 2′-hydroxyl.

DNA polymerases are able to attain an extraordinary high fidelity of nucleotide insertion by combining at least two approaches to distinguish between a correct vs an incorrect nucleotide. First, noncomplementary nucleotides bind to the polymerases in the ground state more weakly than a complementary dNTP. One of the best examples of this is the replication enzyme T7 DNA polymerase which shows over a 400-fold difference in the K_d for the complementary vs a noncomplementary nucleotide (*1*). Second, it is believed that only a complementary nucleotide allows a rapid conformational change to form a ternary complex that aligns the nucleotide and 3′-OH of the primer in the correct orientation for the formation of a phosphodiester bond. This conformational rearrangement was first suggested based upon the results of kinetic experiments with the Pol I family DNA polymerases (*2*−*6*). Conformational changes were identified in the catalytic cycles of polymerases from different families, including *E. coli* DNA polymerase I, T4 DNA polymerase, and the mammalian polymerase β, using fluorescence spectroscopy analysis (*7*, *8*). Recent structural studies of DNA polymerases from four different polymerase families have unambiguously demonstrated the occurrence of a large conformational change associated with binding of the nucleotide (reviewed in ref *9*) and suggest that this conformational rearrangement is necessary for the activity of all DNA polymerases.

Although the DNA polymerase I from *E. coli* is among the best studied polymerases (*10*), a crystal structure of this protein in the catalytically competent ternary complex with DNA and a dNTP has not yet been obtained. Available structural information is limited to the complexes of the Klenow fragment (KF)[1] of this polymerase with dTMP (*11*), the editing complex with duplex DNA (*12*, *13*), and with a dNTP and pyrophosphate (*14*). Here we report a tryptic digestion analysis of binary and ternary complexes of this polymerase with DNA and nucleotides that provides important new data regarding the structural changes that occur in these complexes. Consistent with the structural information available for other DNA polymerases as well as with predictions based upon the results of kinetic experiments, a conformational change in the structure of the KF bound to the primer-template in the presence of the complementary nucleotide is detected. The conformational change is also induced, although to much lower extent, by the dideoxy analogue of the next correct nucleotide, but is not detected in the presence of nucleotides with geometry not compatible with the structure of the polymerase active site. These results provide an insight into the mechanism of action of the DNA polymerase and further support the induced fit model for the fidelity of nucleotide insertion.

MATERIALS AND METHODS

Materials. The Klenow fragment of *E. coli* DNA polymerase I (exonuclease free) was purchased from Amersham Pharmacia Biotech. The protein had been overexpressed and

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[1] Abbreviations: KF, Klenow fragment; W/C, Watson−Crick.

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20-mer primer	5'-GACGAAAACGACGGCCACTT-H
22-mer primer	5'-GACGAAAACGACGGCCACTTAT-H
28-mer template	3'-CTGCTT TTGCTGCCGGTGAATAGTAGTG

FIGURE 1: Sequences of the primers and template used in this study. The primers were terminated by 3′-deoxynucleotides as described under Materials and Methods.

purified from a strain carrying the double mutation D355A, E357A which results in about 10^5-fold reduction of endogenous 3′-5′ exonuclease activity (*15*). T4 polynucleotide kinase was also purchased from Amersham Pharmacia Biotech. Trypsin and terminal deoxynucleotide transferase were from Boehringer Mannheim. Oligonucleotides were obtained from Midland Certified Inc. The 22-mer oligonucleotide primer terminated with 3′-deoxy-UMP was from Genosis. dNTPs, ddNTPs, NTPs, and dAMP were purchased either from Amersham Pharmacia Biotech or from Promega, and the source of nucleotides did not affect the results. [γ-^{32}P]-ATP was from ICN Biomedicals.

Synthesis and Purification of the Oligonucleotides. The sequences of oligonucleotides which were used in this study to create model primer-templates are shown in Figure 1. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. The 20-mer and 22-mer primers lacking 3′-OH were obtained by extension of the corresponding 19-mer and 21-mer with ddTMP using terminal deoxynucleotide transferase. The primer (4.2 μM) was incubated with ddTTP (0.1 mM) in 100 mM cacodylate buffer, pH 6.8, containing 1 mM $CoCl_2$, 0.1 mM DTT, and 62.5 units/mL terminal deoxynucleotide transferase. The reaction was carried out at 37 °C for 2 h, and the resulting 20-mer and 22-mer were purified by electrophoresis in a 20% denaturing gel. The absence of a 3′-OH on the primer terminus of the resulting oligonucleotides was confirmed by lack of primer extension using the procedure described previously (*16*).

Tryptic Digestion of KF. The polymerase−DNA complexes were formed in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 1 mM dithiothreitol. The binding was carried out at room temperature for 15 min in a 12 μL reaction containing 0.6 μM annealed primer-template, 0.3 μM KF (exo−), and 0.4−10 mM dNTP (if present). Two microliters of trypsin solution in water (15 μg/mL final concentration) was added to each reaction mixture, and the digestion was terminated after 6 s by addition of 6 μL of SDS sample buffer containing 0.125 M Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, and 10 μg/mL bromophenol blue. The samples were loaded on a 10% SDS gel, and the electrophoresis was performed according to standard procedure (*17*). Gels were fixed and silver-stained using the GELCODE Color Silver Stain (Pierce) according to the manufacturer's protocol. To further increase the sensitivity of silver staining and detect polypeptide bands containing less than a nanogram of the material, the gels were washed with water 3 times, and the staining procedure was repeated once or twice. The molecular weights of the proteolytic fragments were estimated by electrophoretic mobility using the Low Molecular Weight Electropforesis Calibration Kit (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

The molecular mechanism by which a DNA polymerase inserts a dNMP opposite a complementary base in the template has been the subject of intense investigation for over 3 decades. This remarkable process must combine elements that make the polymerase incredibly specific, so that the fidelity of nucleotide insertion remains high, while still allowing each of the possible base pair combinations in the active site to be accommodated. It was realized early on that the difference in the free energy of a correct vs incorrect base pair was only enough to account for an accuracy of 1 mistake in less than 150 nucleotides incorporated (*1*), while error frequencies for most polymerases are in the range of $10^{-3}-10^{-5}$ in the absence of proofreading (*18, 19*). Therefore, it is now clear that the polymerase plays an active role in enforcing fidelity, but the exact mechanism of this process is still not understood.

Considerable insight into the DNA polymerase mechanism has resulted from kinetic analyses, spectroscopic studies, and crystal structure determinations of wild-type and mutant enzymes (see refs *1, 9, 10,* and *20* for reviews). From these studies, it is clear that the high fidelity results from at least two steps in this process. First, in the ground state or "open" conformation, the polymerase tends to bind complementary nucleotide better than other nucleotides. Second, binding of the complementary dNTP induces a conformational change in the polymerase structure that subsequently results in the rapid incorporation of the nucleotide into the growing DNA chain via the nucleophilic attack of the 3′-OH on the α-phosphate of the dNTP. This conversion to the closed conformation is the rate-limiting step in the polymerization process, and it is thought that the formation of a catalytically competent closed ternary complex requires that the incoming nucleotide fits in the active site and forms proper Watson−Crick (W/C) base pairs. It is the combination of these two processes, nucleotide binding and the "induced fit" conformational change, that appears to provide the high accuracy observed for the polymerization process. Interestingly, different DNA polymerases seem to rely on each of these steps to different extents. For example, T7 DNA polymerase and HIV reverse transcriptase both bind the complementary nucleotide several 100-fold better than incorrect ones (reviewed in ref *1*) while DNA polymerase I has very little selectivity in the ground-state binding (*21*) and therefore must rely mostly on the mechanism of geometric selection for fidelity.

Detection of a Conformational Change Using Trypsin Digestion. Recently we have shown (*16*) that binding of KF (exo$^-$) to a primer-template becomes stronger in the presence of a complementary dNTP and predicted that this was due to the conformational change that formed a closed complex. We also found that the KF−DNA binding becomes weaker in the presence of an incorrect dNTP and proposed that the attempt by the polymerase to accommodate an incorrect nucleotide in the active site leads to an unstable ternary complex. In the present study, we have attempted to detect this presumed conformational change by probing the structure of KF using a limited tryptic digestion analysis. Although tryptic digestion analysis has been previously used to probe structures of herpes simplex virus DNA polymerase and (*22*) and DNA polymerase β bound to different DNA substrates (*23*), it has never been employed to study the influence of the dNTP on the conformation of the polymerase. For our studies, the polymerase was equilibrated with the primer-template in the presence or absence of dNTPs, and the

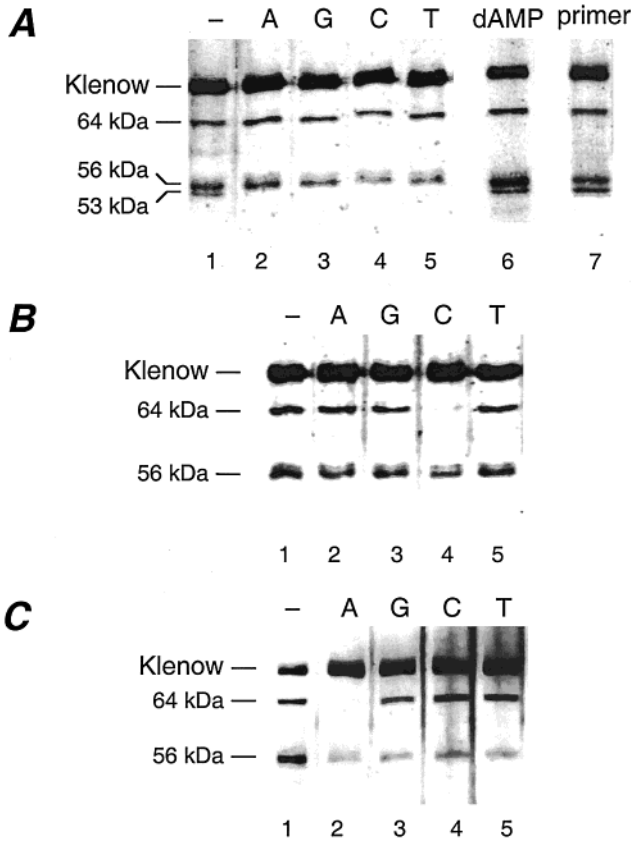

FIGURE 2: Polyacrylamide gel demonstrating the results of tryptic digestion of KF in the presence of the primer-template and/or dNTPs. KF (0.3 μM) was incubated with the primer-template (0.6 μM) and/or dNTPs (10 mM) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described under Materials and Methods. Lanes 1−5: tryptic digestion in the absence of dNTP (lane 1) and in the presence of dATP, dGTP, dCTP, and dTTP (lanes 2−5, respectively). Panel A, no primer-template is present; panel B, the 22/28-mer primer-template is present, making the dCTP the next correct nucleotide; panel C, the 20/28-mer primer-template is present, making the dATP the next correct nucleotide. Lanes 6 and 7 in panel A represent the results of tryptic digestion of KF (0.3 μM) in the presence of dAMP (10 mM) and single-stranded 20-mer (0.6 μM) correspondingly.

conformation of the resulting complex was probed by the analysis of the bands produced following proteolysis (Figure 2). Because it was important to be able to study this complex in the presence of nucleoside triphosphates, the primer used in the current study (Figure 1) was terminated with a 3′-dideoxy sugar, thus preventing the insertion of a nucleotide and allowing for the formation of a ternary polymerase−DNA−dNTP complex.

The results of limited proteolysis of KF in the presence or absence of each of the dNTPs are shown in Figure 2A, lanes 1−5. Without primer-template or nucleotide present, three major peptide fragments are generated having molecular masses of approximately 64, 56, and 53 kDa (Figure 2A, lane 1). When any dNTP is present, the band at 53 kDa is no longer observed (Figure 2A, lanes 2−5), and this effect is not detected in the presense of dAMP as expected (Figure 2A, lane 6) since nucleotide mono- and diphosphates are known not to bind to the polymerase active site (*19*). Similarly, in the presence of a primer-template, the 53 kDa band is no longer observed, even in the absence of a dNTP (Figure 2B, lane 1). The presence of the primer alone does not inhibit the cleavage that generates the 53 kDa band (Figure 2A, lane 7), suggesting that the DNA-induced inhibition is not the result of nonspecific interactions of the positively charged amino acids with polyphosphate backbone of the DNA.

Because lysine and arginine are the basic amino acids that participate in dNTP binding in the KF active site (*14*) and are also the positions in a protein chain cleaved by trypsin (*24*), it is possible that cleavage at the 53 kDa position is blocked by an interaction with the nucleotide triphosphate. The primer-template may also protect this same site from cleavage. However, based upon the available structural information for DNA-bound and free Taq DNA polymerase, it is also possible that the lack of cleavage at this position induced by the primer-template is the result of the structural change that is known to occur upon binding to DNA (*25*).

In the presence of a primer-template and dCTP, which is the nucleotide complementary to the template base in the polymerase active site, the cleavage that produces the band at 64 kDa is now almost completely inhibited (Figure 2B, lane 4). This inhibition does not occur in the presence of an incorrect nucleotide (Figure 2B, lanes 2, 3, and 5). Because the dissociation constants for the interaction of DNA polymerase I with both correct and incorrect nucleotides (and ribonucleotides) are all approximately the same (*21, 26*) and thus the polymerase does not discriminate between nucleotide substrates at the stage of ground-state binding (*1*), it is likely that this inhibition is the result of the conformational change that is known to occur upon the formation of a ternary complex just prior to nucleotide insertion and is not the result of a blockage of the cleavage site by the nucleotide in the open conformation.

Support for the occurrence of the three conformations that are implied by these protease studes has been shown in recent structural studies of the large fragment of *Thermus aquaticus* DNA polymerase I (Klentaq1) (*25*), whose structure closely resembles the *E. coli* KF. These studies reveal the existence of one structure corresponding to the enzyme in the absence of substrates, a second that occurs upon binding DNA, in which the thumb domain of the polymerase almost completely surrounds the DNA, and finally a third complex that is triggered by the binding of the complementary nucleotide resulting from the shift of the fingers domain and leading to formation of the closed ternary complex. The formation of three different patterns of cleavage by trypsin proteolysis is consistent with these structural data (*25*).

Trypsin Cleavage Site. If the inhibition of tryptic digestion at the 64 kDa site in the presence of a complementary dNTP is due to a conformational change in the structure of the KF, it is likely that the cleavage site is in a region of the polymerase molecule that is accessible for trypsin in the open form but becomes inaccessible in the closed form. Although the exact location of the cleavage site is yet to be determined, reasonable predictions can be made about its positioning from knowledge of the amino acid sequence and the three-dimensional structure of the KF. We have used SDS−PAGE analysis and molecular mass standards to estimate the molecular mass of the fragment whose formation is inhibited by the conformational change to be 64 kDa. Assuming that this product is the result of a single cleavage, a reasonable presumption since limiting amounts of trypsin have been used in these experiments and most of the KF is uncut, then about 30−40 amino acids must have been cleaved from the KF to

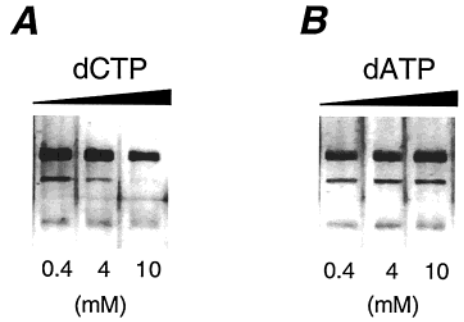

FIGURE 3: Tryptic digestion of KF bound to the 22/28-mer primer-template in the presence of increasing concentrations of nucleotides. KF (0.3 μM) was incubated with the primer-template (0.6 μM) and dNTPs (0.4−10 mM) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described under Materials and Methods. Panel A, dCTP; panel B, dATP.

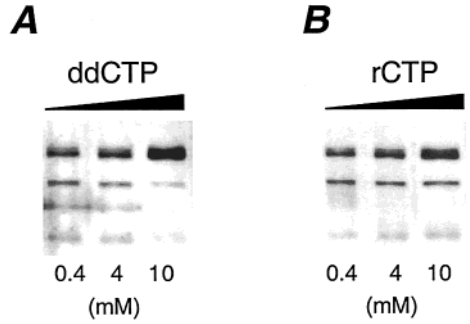

FIGURE 4: Tryptic digestion of KF in the presence of increasing concentrations of ddCTP and rCTP. KF (0.3 μM) was incubated with the 22/28-mer primer-template (0.6 μM) and nucleotides (0.4−10 mM) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described under Materials and Methods. Panel A, ddCTP; panel B, rCTP.

generate the 64 kDa peptide. Removal of this segment from the N-terminus would place the cleavage site in the exonuclease domain of the enzyme. However, there are no lysines or arginines, the amino acids required for trypsin cleavage (*24*), in this region of the enzyme, suggesting that the cleavage site is not in the exonuclease domain. Removal of this length from the C-terminus would place the cleavage site within β-strands 12 or 13 or in the R helix of the DNA polymerase I, which are part of or very close to the polymerase active site. There are four possible trypsin cleavage sites within this region, and based on the structural information available for the open and closed conformations of KlenTaq (*25*), it is reasonable that this region of KF is available for tryptic digestion only when the polymerase is in the open form.

A Complementary dNTP Is Required for the Formation of the Closed Ternary Complex. Several experiments were carried out to support the conclusion that the inhibition of the trypsin cleavage that resulted in the 64 kDa fragment was caused specifically by the presence of the complementary nucleotide. First we determined that this inhibition is not the result of some special interaction with dCTP because when a second primer-template was used (20/28-mer), where dATP is the next correct nucleotide, the 64 kDa band is now missing only in the presence of dATP (Figure 2C). Second, experiments were carried out with the 22/28-mer primer-template in which the nucleotide concentrations of the complementary nucleotide (dCTP) and a noncomplementary nucleotide (dATP) were varied. As shown in Figure 3, panels A and B, the intensity of the cleavage at this position decreases with increasing levels of dCTP (Figure 3A), but not dATP (Figure 3B) or other dNTPs (data not shown). Taken together, these results suggest that the conformational change that presumably is being detected by trypsin cleavage is dependent on the presence of the complementary nucleotide. Providing that the change of the tryptic digestion pattern reflects a change in the conformation of the polymerase, it is likely that the change that is detected only in the presence of the next correct nucleotide is the formation of a stable ternary complex.

Effect of Dideoxy- and Ribonucleotides on Trypsin Cleavage. The "induced fit" theory for polymerase selectivity suggests that the specificity of nucleotide insertion requires that the geometry of the active site formed in the closed structure must correspond to the geometry of the substrate. Recent studies of the incorporation of non-hydrogen-bonding steric analogues of normal W/C nucleotides by Kool and co-workers have demonstrated that steric complementarity is a very important factor in the fidelity of DNA synthesis (*27*, *28*). A logical conclusion from this theory is that the more the shape of the substrate resembles the shape of the active site, the better able is the polymerase to form a closed ternary complex. To test this idea, tryptic digestion was carried out using ddCTP (Figure 4A) and rCTP (Figure 4B), nucleotides that closely resemble the natural substrate for this position, dCTP.

The structural basis for discrimination between a deoxy-, dideoxy-, and ribonucleotide by the KF has been studied by Joyce and co-workers (*26*, *29*, *30*). It has been show that selection against both dideoxynucleotides and ribonucleotides takes place in the transition state for the conformational change that precedes phosphoryl transfer. However, the factors that are responsible for this selection are different. It has been proposed that the polymerase discriminates against rNTPs by steric exclusion during the transition state and that Glu-710 is the "steric gate" that obstructs an incoming rNTP. Steric factors, however, cannot be responsible for discrimination against ddNTPs, which are smaller than the normal substrate. Instead, it was proposed that the 3′-OH of the dNTP, together with other participants, such as the Phe 762 side chain and a magnesium ion, sets up a three-dimensional arrangement that promotes the conformational change, and that the change is impeded when the 3′-OH of the nucleotide is absent. The 3′-OH of the nucleotide plays a role in the correct positioning of the nucleotide for the nucleophilic attack, but, as judged by sulfur elemental effects, the rate of the chemistry step is not significantly lower for ddNTP (26). This suggests that the ddNTP can be positioned in the active site to allow phosphodiester bond formation but that the rate constant for conformational change is much lower than obtained with a dNTP. Thus, these prior studies indicate that formation of a stable ternary complex with correct geometry is impossible with the rNTPs because of steric constraints, but is allowed with ddNTPs, although with a much lower rate.

This prediction is supported by the results of the protease digestion. When the nucleotide used is ddCTP (Figure 4A), the formation of the closed complex is allowed, although at nucleotide concentrations higher than for dCTP (Figure 3A).

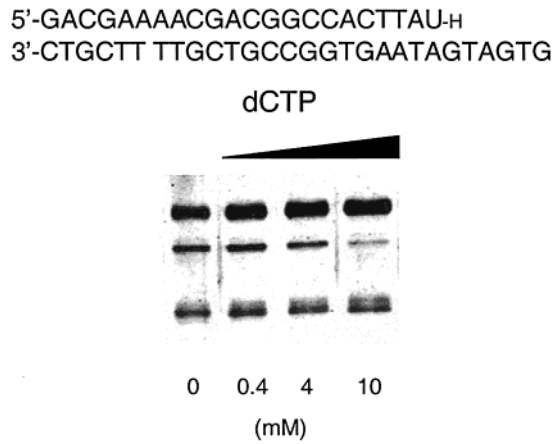

FIGURE 5: Tryptic digestion of KF bound to the 22/28-mer primer-template containing 3′-deoxyuridine at the 3′ end of the primer. KF (0.3 μM) was incubated with the primer-template (0.6 μM) and dCTP (0−10 mM) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described under Materials and Methods.

On the other hand, a stable closed complex is not formed with rCTP (Figure 4B) at the highest concentration tested (nucleotide concentrations higher than 15 mM lead to inhibition of tryptic digestion at all sites independently from the nature of the nucleotide and/or the presence of DNA and therefore were not used in this study).

A 2′-OH at the Primer Terminus Inhibits the Conformational Change. According to the "induced fit" model for nucleotide incorporation, not only the structure of the incoming nucleotide but also the structure of the polymerase active site formed by both the protein and the DNA is critical for successful insertion of a nucleotide. To test this idea and to further characterize the detected conformational change, the tryptic digestion experiments were carried out on complexes of KF with the primer-template containing 3′-deoxyuridine at the 3′ end of the primer (i.e., containing a 2′-OH). Comparison of the results of this experiment (Figure 5) with the cleavage patterns on the same primer-template lacking the 2′-OH (Figure 3A) suggests that the 2′-hydroxyl of the primer has a destabilizing effect on the formation of the closed ternary complex. This is consistent with the observation that a primer containing ribonucleotides can be extended by polymerase I, although the rate of DNA synthesis from this type of primer is reduced by 1 or 2 orders of magnitude (*19*). Positioning of 3′-dUMP instead of ddTMP at the 3′ end of the primer may result not only in steric interference within the active site because of the 2′-OH, but possibly in more global changes in the structure of the DNA. For instance, it is known that a ribonucleotide can affect the equilibrium between A-like and B-like conformations of the helix (*26*). The results of the tryptic digestion experiments reported here imply that the catalytic complex formed by KF bound to this altered primer terminus is less capable of accommodating the incoming nucleotide to form the closed ternary complex.

Conclusions. In this study we have used limited protease digestions to detect the conformational changes that occur when *E. coli* DNA polymerase I (Klenow fragment) binds to DNA and nucleotide substrates. These results strongly support the idea that a close match between the geometric shape of the substrate and the polymerase active site is critical for the production of the conformational rearrangement required for nucleotide insertion.

It is well established that the chemical step rather than a conformational change becomes rate-limiting when the polymerase is incorporating an incorrect dNTP (*3*). However, these prior studies do not explain the effect of a non-W/C nucleotide on the ternary complex. The implications of the results from this study and our prior observations (*16*) suggest that although the presence of a non-W/C nucleotide may induce a conformational change in the polymerase, this change does not lead to the formation of a stable catalytically competent ternary complex. Instead, it seems to destabilize the polymerase−DNA interactions and increase the probability of polymerase dissociation from DNA. This mechanism may account for both high fidelity and low processivity of DNA polymerase I.

The method described here also provides a useful tool for detecting a conformational change in *E. coli* DNA polymerase I and possibly in other polymerases in response to not only the nucleotide substrates but also other factors, such as DNA substrate, buffer, salts, or metal ions, and other proteins. Moreover, this simple approach can also be used to obtain information about the structure of a polymerase active site and the molecular mechanism of mutagenesis and adduct-induced inhibition of DNA replication. For example, using these methods, we have recently demonstrated that the distorting carcinogenic DNA adduct *N*-(2′-deoxyguanosin-8-yl)-*2*-acetylaminofluorene (dG-AAF) interferes with the conformational change of DNA polymerase I (but not the primer-template binding), while the related but less distorting and easily bypassed deacetylated derivative *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene (dG-AF) has little effect on the conformational change (*31*).

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