Presence of 18-Å Long Hydrogen Bond Track in the Active Site of Escherichia coli DNA Polymerase I (Klenow Fragment)

ITS REQUIREMENT IN THE STABILIZATION OF ENZYME-TEMPLATE-PRIMER COMPLEX*

Received for publication, November 11, 2002, and in revised form, December 23, 2002
Published, JBC Papers in Press, January 9, 2003, DOI 10.1074/jbc.M211496200

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The analysis of the active site region in the crystal structures of template-primer-bound Klenow fragment equivalent of Thermus aquaticus DNA polymerase I shows the presence of a 18-Å long H-bonding track contributed by the Klenow fragment equivalent of Asn845, Gln849, Arg668, His881, and Gln677. Its location is nearly diagonal to the helical axis of the template-primer. Four base pairs in the double stranded region proximal to 3' OH end of the primer terminus appear to interact with individual amino acid components of the track through either the bases or sugar moieties. To understand the functional significance of this H-bonding network in the catalytic function of Klenow fragment (KF), we generated N845A, N845Q, Q849A, Q849N, R668A, H881A, H881V, Q677A, and Q677N mutant species by site-directed mutagenesis. All of the mutant enzymes showed low catalytic activity. The kinetic analysis of mutant enzymes indicated that $K_m$ for dNTP was not significantly altered, but $K_p$ for DNA was significantly increased. Thus the mutant enzymes of the H-bonding track residues had decreased affinity for template-primer, although the extent of decrease was variable. Most interestingly, even the reduced binding of TP by the mutant enzymes occurs in the nonproductive mode. These results demonstrate that an H-bonding track is necessary for the binding of template-primer in the catalytically competent orientation in the pol I family of enzymes.

The examination of the interactive environment of individual residues of this track further clarifies the mode of cooperation in various functional domains of pol I.

Sequence alignment of various nucleic acid polymerases has revealed the presence of several conserved motifs (motifs A–E) in diverse DNA polymerase families (1–3). Two of these motifs (motifs A and C) are absolutely conserved in all DNA polymerase families, whereas the conservation of other motifs is restricted to individual families. Two conserved aspartates, one belonging, respectively, to motifs A and C each, serve as ligands for divalent cations (4–6) and tags for the active site location of motifs A and C resides at the palm subdomain, the dNTP binding site at the fingers subdomain, and the template-primer binding site at the thumb subdomain (7).

The crystal structures of several DNA polymerases complexed with template-primer and dNTP have shown the participation of individual amino acid residues in the respective polymerase function (4, 6, 11, 12). In particular, the crystal structures of binary and ternary complexes of the Klenow fragment of Thermus aquaticus DNA polymerase I (KlenTaq) can be considered to represent the snapshots of initial steps of the nucleotidyltransferase reaction pathway of DNA polymerases (11). The analyses of these crystal structures together with the crystal structure of apo-KlenTaq revealed that the binding of template-primer to the enzyme induces a conformational rearrangement of the side chains of several residues present at the active site. We noted that resulting from this conformational change in the side chains is the formation of a predominately hydrogen bonded network of ~18 Å at the bottom of the cleft (Fig. 1). This network of hydrogen bonding (called “H-bonding track” hereafter) is present in both binary and in the “open” and “closed” conformations of the ternary complexes.

The residues that participate in the formation of the H-bond track are highly conserved in the pol I family of DNA polymerases. They are equivalent to the E. coli pol I residues Asn645, Gln649, Arg668, His681, and Gln677. To understand the significance of the H-bonding track residues at the active site of pol I and their functional contribution, we carried out site-directed mutagenesis of Asn645, Gln649, Arg668, His681, and Gln677. These residues were mutated to both conserved and nonconserved (alanine) residues. Properties of the mutant proteins were then investigated to assess their individual participation in the catalytic function of KF. All of the mutants showed decreased catalytic activity, albeit to different degrees, suggesting the involvement of these residues in the catalytic function of pol I. Most importantly, all of the

* This work was supported in part by National Institutes of Health NIGMS Grant GM 36307. 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.
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1 The abbreviations used are: KF, Klenow fragment of E. coli DNA polymerase I; pol I, E. coli DNA polymerase I; DTT, dithiothreitol; TP, template-primer; KlenTaq, Klenow fragment equivalent of T. aquaticus DNA polymerase I; Bet, Klenow fragment equivalent of B. stearothermophilus DNA polymerase I; WT, wild-type.

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mutant enzymes showed significantly reduced (15–75-fold) template-primer binding affinity compared with wild-type enzyme. Furthermore, neither the conservative nor the non-conservative mutant enzymes showed ability to form prepolymerase binary or ternary complexes. Similarly, unlike the wild-type enzyme, the enzyme-TP covalent complexes generated for individual mutant species of all five residues failed to catalyze in vitro DNA synthesis. These results further with the structural analysis of the binary and ternary complexes of pol I family DNA polymerases strongly implicate the function of H-bonding track residues in the binding of template-primer at the proper position and orientation, which is prerequisite for the nucleotidyltransferase function of polymerases. An examination of the location of some of the highly conserved residues present in different motifs of pol I, in the context of the H-bonding track, further reveals the coordinated participation of all of these motifs in the catalytic function.

EXPERIMENTAL PROCEDURES

Materials—All mutant enzymes were generated from the plasmid (pCJ141) generously provided by Dr. Catherine Joyce of Yale University. This construct contains the \( E. \ coli \) polA gene encoding the Klenow fragment (13). The maintenance and expression strains for plasmid pCJ141 (E. coli Cj406 and E. coli Cj376, respectively) were also obtained from Dr. Joyce.

Enzymes—PFU-turbo polymerase used for PCR-based site-directed mutagenesis was from Stratagene. Restriction enzymes were from Roche Molecular Biochemicals. Polynucleotide kinase from either Invitrogen Corp. or PerkinElmer Life Sciences was used.

Reagents—The PCR grade dNTPs were from Roche Molecular Biochemicals. Radiolabeled nucleotides were obtained from PerkinElmer Life Sciences. The DNA extraction kit was from Qiagen, whereas DNA oligonucleotides were from MWG-Biotechnologies. All 32P-labeled oligomers were purified by denaturing polyacrylamide-urea gel electrophoresis.

In Vitro Site-directed Mutagenesis—We used the PCR-based protocol described in Stratagene’s QuickChange site-directed mutagenesis kit to generate the desired mutations of KF. The plasmid pCJ141 (13, 14) was used for the generation of KP protein and to construct the desired H-bonding track mutant derivative. This plasmid contains the \( E. \ coli \) KP gene carrying a mutation, D424A. This mutation confers deficiency in 3′-5′ exonuclease activity. The wild-type KP used for this study and its Asn454, Glu464, Arg484, His484, and Gln574 mutants are therefore exonuclease-deficient. All mutations were confirmed by DNA sequencing.

Expression and Purification of the Wild-type and Mutant Proteins—Isolation and purification of N845A, N845Q, Q849A, Q849N, R868A, H881A, H881V, Q677A, and Q677N mutant enzymes were carried out as described before (15). Plasmid DNA from mutant clones was transfected E. coli Cj376, an expression strain used for this study (13, 16).

Briefly, an overnight inoculum of the expression strain was used to initiate a 500-mL cell culture at 30 °C, in an incubator shaker. At a OD600 of 0.3, the incubation temperature was raised to 42 °C to heat induce overproduction of the enzyme. After 4–5 h of incubation, cells were harvested, washed, and resuspended in cell lysis buffer (50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) containing 2 mM MgCl2. Following a 30-min incubation at 4 °C, the cell suspension was sonicated and centrifuged (14,000 rpm for 30 min), and the supernatant was passed through a DEAE column to remove DNA. The flow-through was fractionated with ammonium sulfate, using 60 and 85% saturations. The pellet obtained with 85% ammonium sulfate was resuspended in 5 mL of buffer B (50 mM Tris-Cl, pH 7.0, 1 mM dithiothreitol (DTT), 1 mM EDTA) and dialyzed overnight against 1 liter of the same buffer, and applied to a Bio-Rex 70 column prewashed with Buffer B. At 500 mM linear gradient of NaCl in Buffer I was used to elute the bound protein. Peak fractions (representing a 68-kDa protein on SDS-polyacrylamide gel electrophoresis) were pooled and concentrated with polyethylene glycol (8000). The samples were further dialyzed in a buffer containing 50 mM Tris-Cl, pH 7.0, 1 mM DTT, 100 mM NaCl. Protein concentrations were determined by the Bradford colorimetric assay (17) and the enzyme stocks (in 20% glycerol) were stored at 20 °C.

Specific Activity Determination—Enzymatic activity of the various mutants was determined at 37 °C for 5 min on one heteropolymeric (49/17-mer) and two homopolymeric (dA12/ddC9, dC32/ddG32) template-primers (Chart 1). The reaction was carried out in a final volume of 100 μL containing 50 mM Tris-Cl, pH 7.8, 1 mM DTT, 100 μg/ml bovine serum albumin, 250 nM of the template-primer, 5 mM MgCl2, and 25 μM [32P]dNTP (0.5 μCi assay) corresponding to the homo- and heteropolymeric template-primers. Reactions with the heteropolymeric template-primer contained all four dNTPs at a concentration of 25 μM, with two of them radiolabeled. Unless otherwise indicated, the final enzyme concentration was 7.5 nM. The reaction was initiated by the addition of MgCl2 and terminated by the addition of 5% ice-cold trichloroacetic acid containing 10 mM PP. The acid-precipitated material was collected on Whatman GF/F filters and counted for radioactivity in a liquid scintillation counter, as described elsewhere (18).

Template-primer for with in situ nucleotide incorporation was prepared as described previously (19). The binding of 33/20 + dC-mer and 21/12 + dC, the deoxy-terminated template-primers (Chart 1), to various concentrations of enzyme was carried out in a reaction mixture containing 50 mM Tris-Cl, pH 7.8, 5 mM MgCl2, 10% (v/v) glycerol, and 0.1 mg/ml bovine serum albumin. The concentration of 32P-labeled template/primer was 50–100 pmol. Different protein concentrations were used to bracket the \( K_{D_{DNA}} \) value. Samples were electrophoresed at 100 V for 1.5 h at 4 °C on a 6% nondenaturing polyacrylamide gel, using 89 mM Tris borate, pH 8.2, buffer. Gels were dried, tractions were used to bracket the \( K_{D_{DNA}} \) value. Samples were electrophoresed at 100 V for 1.5 h at 4 °C on a 6% nondenaturing polyacrylamide gel, using 89 mM Tris borate, pH 8.2, buffer. Gels were dried, tractions were used to bracket the \( K_{D_{DNA}} \) value. Samples were electrophoresed at 100 V for 1.5 h at 4 °C on a 6% nondenaturing polyacrylamide gel, using 89 mM Tris borate, pH 8.2, buffer. Gels were dried, tractions were used to bracket the \( K_{D_{DNA}} \) value. Samples were electrophoresed at 100 V for 1.5 h at 4 °C on a 6% nondenaturing polyacrylamide gel, using 89 mM Tris borate, pH 8.2, buffer. Gels were dried, tractions were used to bracket the \( K_{D_{DNA}} \) value. Samples were electrophoresed at 100 V for 1.5 h at 4 °C on a 6% nondenaturing polyacrylamide gel, using 89 mM Tris borate, pH 8.2, buffer. Gels were dried, tractions were used to bracket the \( K_{D_{DNA}} \) value.
induce ternary complex formation. The stability of the enzyme-TP binary complex and enzyme-TP-dNTP ternary complex was then assessed by the degree of persistence of DNA in the enzyme-TP complex, upon addition of 500-fold excess of the same unlabeled template-primer. We find that under these conditions, DNA from enzyme-TP binary complexes completely dissociates, whereas ternary complexes exhibit significant retention of radiolabeled template-primer by enzyme protein. All samples were resolved on a native 6% polyacrylamide gel, and the positions of the radioactive bands representing enzyme-DNA and free DNA were visualized by exposure in a PhosphorImager.

Effect of Complementary or Noncomplementary Incoming Nucleotide on Template-primer Binding Affinity—The effect of the complementary and noncomplementary nucleotide on the binding affinity of wild-type and mutant enzymes was assessed in a manner similar to that used for the stable ternary complex formation assay. The enzyme-TP binary complexes were allowed to form in 50 mM Tris-HCl, pH 7.8, 10% (v/v) glycerol, 5 mM MgCl2, and 0.1 mg/ml bovine serum albumin for 10 min at 4 °C in duplicate. To one of the duplicate samples, the correct incoming dNTP (200 μM dGTP) was added. The incorrect dNTPs (dATP, dCTP, and dTTP, 100 μM each) were added to the second sample. The reaction mixture was further incubated on ice for 10 min, prior to loading on a 6% nondenaturing polyacrylamide gel, under the same conditions as those used for KD,DNA determination.

Enzyme-Template-primer Cross-linking—The UV-mediated photochemical enzyme-TP cross-linking was performed essentially as described elsewhere (18, 22, 23). The reaction mixtures containing 50 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 1 mM DTT, and 5 mM CTP, 50 mM Tris-HCl, pH 7.8, 1 mM DTT, and 5 mM MgCl2. The reaction contained 30 pmol of the wild-type KF, whereas the amount of individual mutant enzymes was varied to yield near equal quantities of enzyme-TP cross-linked species. The nucleotidyltransferase reactions were then initiated by the addition of 1 nM NaCl together with 5 μCl of complementary [α-32P]dATP at a final concentration of 0.5 μM. The reaction mixture was incubated for 30 min at room temperature and terminated by the addition of protein solubilizing solution. An aliquot of the reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis, followed by the exposure of gel to PhosphorImager and analysis of exposed gel by ImageQuant software (Amersham Biosciences).

RESULTS

The structural analysis of the template-primer and template-primer-dNTP bound crystal structures of KlenTaq and Bacillus steaothermophilus (11, 24) showed the presence of a network of interactions (mainly H-bonds) near the active site of these polymerases. The constituent residues in these two enzymes (KlenTaq and Bst) are equivalent to Asn845, Gln849, Arg668, His863, and Gln677 of E. coli DNA polymerase I, which participate in the formation of H-bonding track (shown as broken cylinder in gray color) in the Klew fragment of Taq polymerase. This figure has been generated from the crystal structure of the enzyme-TP binary complex of KlenTaq (11) (Protein Data Bank file 4ktq). In panel A, the phosphate backbone of the template (blue) and primer (gold) are shown as ribbons. The constituent amino acid residues of the H-bonding track are colored in cyan and are shown as sticks. The two metals (in magenta balls marked A and B) at the active site and two essential aspartic acids (shown as green sticks) as seen in the ternary complex of KlenTaq have also been included as reference points. Because Asp667 of KlenTaq (equivalent to Asp597 of KF) assumes two different conformations in the binary and ternary complexes, its orientation in this figure is as seen in the "closed ternary complex" crystal structure (Protein Data Bank file 3ktq). The dotted lines among H-bonding track residues represent the hydrogen bond. An extra pink dotted line between Arg668 and His863 equivalent residues of KlenTaq represents the van der Waals interactions between these two side chains. The potential donor atoms of H-bonding track residues that can form the hydrogen bond with the minor groove moieties of template-primer (purine N3 and pyrimidine C = O groups) are shown as blue balls. The amino acid residues and positions in KlenTaq are labeled with KF numbers based on their structural and positional equivalence. In panel B, a stereo-diagram depicting another view of the H-bonding track residues is shown. This view has been generated by rotating the figure in panel A by about 90° in the clockwise direction. In this figure, the template and primer are colored blue and gold, respectively. The bonds of the template and primer are rendered in sticks, whereas the bases and sugar moieties have been filled by plates. The amino acid residues, rendered in sticks, are also shown with their van der Waals surface in different colors. These figures were generated by MolMol (44).

Fig. 1. Presence of an 18-Å long H-bonding track formed by five amino acid residues of KlenTaq. This figure shows the positions of five amino acid residues equivalent to Asn845, Gln849, Arg668, His863, and Gln677 of E. coli DNA polymerase I, which participate in the formation of H-bonding track (shown as broken cylinder in gray color) in the Klew fragment of Taq polymerase. This figure has been generated from the crystal structure of the enzyme-TP binary complex of KlenTaq (11) (Protein Data Bank file 4ktq). In panel A, the phosphate backbone of the template (blue) and primer (gold) are shown as ribbons. The constituent amino acid residues of the H-bonding track are colored in cyan and are shown as sticks. The two metals (in magenta balls marked A and B) at the active site and two essential aspartic acids (shown as green sticks) as seen in the ternary complex of KlenTaq have also been included as reference points. Because Asp667 of KlenTaq (equivalent to Asp597 of KF) assumes two different conformations in the binary and ternary complexes, its orientation in this figure is as seen in the "closed ternary complex" crystal structure (Protein Data Bank file 3ktq). The dotted lines among H-bonding track residues represent the hydrogen bond. An extra pink dotted line between Arg668 and His863 equivalent residues of KlenTaq represents the van der Waals interactions between these two side chains. The potential donor atoms of H-bonding track residues that can form the hydrogen bond with the minor groove moieties of template-primer (purine N3 and pyrimidine C = O groups) are shown as blue balls. The amino acid residues and positions in KlenTaq are labeled with KF numbers based on their structural and positional equivalence. In panel B, a stereo-diagram depicting another view of the H-bonding track residues is shown. This view has been generated by rotating the figure in panel A by about 90° in the clockwise direction. In this figure, the template and primer are colored blue and gold, respectively. The bonds of the template and primer are rendered in sticks, whereas the bases and sugar moieties have been filled by plates. The amino acid residues, rendered in sticks, are also shown with their van der Waals surface in different colors. These figures were generated by MolMol (44).
As described under “Experimental Procedures,” DNA polymerization assays were carried out under standard conditions with the indicated template/primer. The values are expressed relative to the wild-type and were performed using Mg²⁺ as the divalent cation. One hundred percent activity of KF with \( \text{dA}_36/\text{dT}_{18} \) and \( \text{dC}_60/\text{dG}_{18} \) was \( 1.10 \times 10^{5} \) units/mg protein, respectively. For the 49/18-mer template/primer, 100% activity of KF equals \( 1.21 \times 10^{4} \) units/mg of protein. One unit is defined as the activity necessary to incorporate 1 nmol of dNMP into acid-insoluble material in 30 min at 37°C.

### Table I: Relative specific activity of mutant enzymes of H-bonding track in KF

| Enzyme     | Template-primer | 49/18-mer |
|------------|-----------------|-----------|
|            | \( \text{dA}_36/\text{dT}_{18} \) | \( \text{dC}_60/\text{dG}_{18} \) |           |
| WT         | 100             | 100       | 100       |
| N845A      | 10              | 14        | 20        |
| N845Q      | 1               | 2         | 1         |
| Q677A      | 1               | 15        | 16        |
| R668A      | 0.2             | 1         | 19        |
| Q849N      | 2               | 9         | 1         |
| H881A      | 16              | 26        | 24        |
| H881V      | 21              | 29        | 24        |
| Q677T      | 11              | 32        | 21        |
| Q677N      | 2               | 8         | 2         |

The polymerase activity of mutant enzymes was determined with two homopolymeric (\( \text{dA}_36/\text{dT}_{18} \) and \( \text{dC}_60/\text{dG}_{18} \)) template/primer and in situ addition, the WT and H-bonding track mutant proteins also contained the D424A mutation at the 3′−5′ exonuclease active site, rendering them exonuclease-deficient. Thus, the mutant protein D424A is referred to as WT and R668A (also containing D424A mutant) as R668A mutant for the polymerase activity studies.

Specific Activities of the Mutant Enzymes with Different Template-primers—The polymerase activity of mutant enzymes was determined with two homopolymeric (\( \text{dA}_36/\text{dT}_{18} \) and \( \text{dC}_60/\text{dG}_{18} \)) and one heteropolymeric (49/17-mer) template/primer in the presence of Mg²⁺ as metal cofactor. The activities of different mutant enzymes expressed as a percentage of WT are summarized in Table I. All mutants showed a significantly decreased activity with \( \text{dA}_36/\text{dT}_{18} \) ranging between 0.2 and 32% of the level in the wild-type enzyme. Generally, the two His⁶⁸¹ mutants (H881A and H881V) appeared to retain relatively higher activity with all three template-primers, namely \( \text{dA}_36/\text{dT}_{18} \), \( \text{dC}_60/\text{dG}_{18} \) (homopolymers), and 49/17-mer (heteropolymer). Among the three template-primers, the least activity of individual mutant enzymes was seen with \( \text{dA}_36/\text{dT}_{18} \). The most severe effect on the catalytic activity was noted with the N845Q, Q849N, R668A, and Q677N mutant species of KF.

Surprisingly, the conserved substitutions of residues Asn⁶⁴⁵, Gln⁶⁴⁹, and Gln⁶⁷⁷ consistently exhibited greater loss of activity with all of the template-primers, compared with nonconserved substitutes at these positions. These activity data suggested that the side chains of Asn⁶⁴⁵, Gln⁶⁴⁹, Arg⁶⁶⁸, His⁶⁸¹, and Gln⁶⁷⁷ have some important role in the polymerase function of KF.

## Determination of Steady-state Kinetics Constants

The steady-state kinetic parameters, \( k_{\text{cat}} \) for the polymerase reaction and \( K_m \) for dNTP utilization, were determined for the WT and each mutant protein, with two homopolymeric DNA substrates (\( \text{dA}_36/\text{dT}_{18} \) and \( \text{dC}_60/\text{dG}_{18} \)). The rates of incorporation of \( \alpha^{-32} \text{P}-\text{dNTP} \) into products at increasing concentrations of dNTP were measured, and \( k_{\text{cat}} \) for the polymerase reaction and \( K_m \) for dNTP were determined by Eadie-Hofstee plots from the rate data. The results are summarized in Table II. The wild-type enzyme exhibited a \( K_m \) of \( 5 \mu \text{M} \) with both homopolymeric template-primers. The mutant N845A showed almost no change in \( K_m \) with either template-primer, whereas the catalytic efficiency was decreased by \( 6\)-fold with \( \text{dA}_36/\text{dT}_{18} \) template-primer. The catalytic efficiency with \( \text{dC}_60/\text{dG}_{18} \) template-primer was not changed significantly for the N845A mutant. However, the conserved mutant, N845Q, showed a drastic change of \( 85\)-fold in catalytic efficiency. The mutants of Gln⁶⁴⁹ exhibited a moderate change in \( K_m \) only with \( \text{dC}_60/\text{dG}_{18} \) template-primer (−5–10-fold). However, the steady-state catalytic rate (\( k_{\text{cat}} \)) was significantly reduced with both template-primers (20-fold with \( \text{dA}_36/\text{dT}_{18} \) and 60-fold with \( \text{dC}_60/\text{dG}_{18} \)). The change in both \( K_m \) and \( k_{\text{cat}} \) was reflected in significantly decreased catalytic efficiency (14–75-fold) of Gln⁶⁴⁹ mutants. For both, Asn⁶⁴⁵ and Gln⁶⁴⁹, the greater effect was noted with conserved substitutions suggesting that not only the chemical nature of the side chain but also the size of the side chain at a specific position is critical for KF to function optimally. The two His⁶⁸¹ mutants did not show significant change in \( K_m \); however, the \( k_{\text{cat}} \) of both enzymes (H881A and H881V) was significantly (−65-fold) reduced with \( \text{dA}_36/\text{dT}_{18} \) template-primer. This effect was not as pronounced with \( \text{dC}_60/\text{dG}_{18} \) template-primer, as only a moderate (−6-fold) change was noted in the catalytic rate of both His⁶⁸¹ mutants.

The mutants of Gln⁶⁷⁷ (Q677A and Q677N) also showed a defect in their catalytic efficiency. The efficiency of the Q677A mutant was reduced by \( 10\)- and 120-fold on \( \text{dA}_36/\text{dT}_{18} \) and \( \text{dC}_60/\text{dG}_{18} \) template-primers, respectively. As noted for Asn⁶⁴⁵ and Gln⁶⁴⁹, the efficiency of the homologous mutant of Gln⁶⁷⁷ (Q677N) was significantly more reduced than that of its nonhomologous counterpart (Q677A). The catalytic efficiency was reduced by nearly 3000-fold on \( \text{dA}_36/\text{dT}_{18} \) and by \( 300\)-fold with \( \text{dC}_60/\text{dG}_{18} \) template-primer. Similarly, a reduction of about 300-fold in the catalytic efficiency of the R668A mutant enzyme was noted with \( \text{dA}_36/\text{dT}_{18} \) template-primer. Similar (type of) template-specific inactivation was also noted with the mutants of O-helix residues (18). These steady-state kinetic data revealed that most of the mutant enzymes have a compromised DNA polymerization efficiency, suggesting some defect in the mutant of enzymes at one or more steps of the catalytic mechanism.

### Ability of Mutant Enzymes to Form E-TP Binary Complexes—Because the mutants of Asn⁶⁴⁵, Gln⁶⁴⁹, Arg⁶⁶⁸, His⁶⁸¹, and Gln⁶⁷⁷ amino acid residues of the KF displayed reduced polymerase activity, we examined if this decrease was because of a defect in template-primer binding ability of mutant proteins. To determine the binding affinity of various mutant enzymes with the template-primer, we carried out gel mobility shift assay of the wild-type and mutant enzymes with 33/20 + ddC and 21/12 + ddC, two template-primers that differ in duplex length (Chart 1). The primer moiety of 33/20-32P-radiolabeled, whereas in the 21/12 + ddC template-primer, the template strand was radiolabeled. The individual enzyme-TP complexes were resolved on 6% nondenaturating polyacrylamide gels. The representative patterns of the migration of enzyme-TP complexes for each of the two template-primers (33/20 + ddC and 21/12 + ddC) are
shown in Fig. 2. Panels A and B in this figure show the formation of the enzyme-TP complex between wild-type KF and 33/20 + ddC and 21/12 + ddC template-primers, respectively, with increasing concentrations of the wild-type KF ranging between 0.3 and 20 nM for the 33/20 + ddC and 0.04 nM to 1.24 nM for the 21/12 + ddC. From the comparison of the distribution of radio-labeled species between complexed and uncomplexed positions in panels A and B, it is clear that at 1.24 nM concentration of the wild-type KF, nearly 85% of the 21/12 + ddC template-primer has been stably bound. In contrast, the same amount of enzyme (4th lane from left in panel A of Fig. 2) could bind 55% of the 33/20 + ddC template-primer. These data suggest that the affinity of wild-type KF for a template-primer with a short duplex region (21/12 + ddC with only 13 base pairs) is greater than that for a template-primer containing a longer duplex region (33/20 + ddC with 21 base pairs). The $K_D$ values for 33/20 + ddC and 21/12 + ddC template-primers were 0.60 and 0.14 nM, respectively, calculated by plotting the concentration of enzyme against that of complexed template-primer and fitting the data to the hyperbolic curve shown in Fig. 2, panels C and D. Another interesting observation pertaining to the binding of KF with two template-primers is the gel mobility pattern of the enzyme-TP complex, particularly at high enzyme concentrations. It appears that with increasing concentrations of KF, a regular 1:1 enzyme-TP complex forms first, which is then shifted to a slower migrating species (supershift position) (Fig. 2, panel A). This kind of supershift is not seen with the use of 21/12 + ddC template-primer (Fig. 2, panel B). Note that the last two lanes in each panel show the pattern obtained with 75 and 150 nM enzyme concentration. Thus it appears that the template-primer containing 21 base pairs may support sequential binding of two enzyme molecules, albeit with different affinity.

The template-primer binding affinity of mutant enzymes of Asn$^{445}$, Gln$^{449}$, Arg$^{669}$, His$^{881}$, and Gln$^{677}$ was determined in a similar fashion as described above for the wild-type KF. A representative profile of DNA binding by R668A and Q849N with two template-primers is shown in Fig. 3. The estimated $K_D$ values for various mutant enzymes with 33/20 + ddC template-primer are listed in Table II. It is clear from this table that the template-primer binding affinity of all mutant proteins was significantly decreased. The $K_D$ difference varied in the range of 15–75-fold with this template-primer. The difference in $K_D$ was further increased when measured with 21/12 + ddC template-primer. For example, the $K_D$ for the R668A mutant with this template-primer was ~100-fold greater than that for wild-type enzyme. Curiously, the migration pattern of enzyme-TP complexes formed with the 21-base pair long template-primer by all the mutant enzymes appeared only as the supershifted species (Figs. 3 and 4). Furthermore, no clear band of enzyme-TP complex with the 21/12 + ddC template-primer was observed. Two representative patterns of these effects are shown in Fig. 3. Fig. 3A shows the binding affinity of R668A (left) and Q849N (right) mutants with 33/20 + ddC template-primer. The concentrations of R668A and Q849N ranged between 4.8 and 153.6 nM. It is clear that the enzyme-TP complex is detectable at high enzyme concentrations and at the migration position of the supershifted species. The two lanes (lanes 8 and 9 in the left panel) show the mobilities of the enzyme-TP complex formed between wild-type KF and 33/20 + ddC. In lane 8, the concentration of wild-type KF (4.8 nM) has been chosen such that it shows both (shift and supershift) positions while in lane 9, the concentration is higher (75 nM), which results in the formation of only supershifted species. Similarly, lanes 8–10 in the right panel show the mobility of wild-type enzyme-TP complexes formed with 3.6, 7.2, and 25 nM enzyme and 33/20 + ddC. A transition from shift and supershift position is clearly visible here with this template-primer. The binding of R668A and Q849N mutant KF to 21/12 + ddC template-primer is shown in panel B of Fig. 3. It is clear from the results that both R668A and Q849N mutant enzymes fail to form a distinct species resembling the enzyme-TP complex, although the amount of free TP is continuously decreasing as the concentration of the protein is increased. A shade of gray above the free template-primer for R668A mutant enzyme (possibly a dissociated complex) is nonetheless seen at higher concentrations of the enzyme. This shade of gray, which is not so obvious for the Q849N mutant enzyme, suggests that there are further differences among different mutants with regards to the binding of the shorter duplex containing template-primer. Panel C of Fig. 4 shows curves representing the binding affinity of R668A for 33/20 + ddC and 21/12 + ddC template-primers and Q849N for 33/20 + ddC. The $K_D$ values of the R668A mutant for two template-primers (33/20 + ddC and 21/12 + ddC), estimated from these plots, are 14 and 33 nM, respectively. The $K_D$ of the Q849N mutant enzyme with 33/20 + ddC template-primer is 31 nM. The affinity of Q849N for 21/12 + ddC template-primer appears very low and could not be calculated from these data. These results strongly suggest that there are two binding modes of KF to template-primer and that the mutants of H-bonding track residues bind only in the supershifted mode.

**Effect of Correct and Incorrect dNTPs on E-dNTP Binary Complex**—The other means that we employed to investigate

| Enzyme | $dA_9/dT_{38}$ | $dC_{36}/dG_{18}$ | $K_D$ DNAadj (33/20 + ddC) |
|--------|----------------|------------------|--------------------------|
|        | $K_m$ $k_{cat}$ $k_{cat}/K_m$ | $K_m$ $k_{cat}$ $k_{cat}/K_m$ | $K_D$ DNA |
|        | $\mu M$ $s^{-1}$ $M^{-1} s^{-1}$ | $\mu M$ $s^{-1}$ $M^{-1} s^{-1}$ | $nM$ | Ratio WT/mutant |
| WT     | 7.0 2.0 2.8 $\times 10^5$ | 5.0 6.0 1.2 $\times 10^6$ | 0.6 | 1.0 |
| N845A  | 3.0 0.14 0.5 $\times 10^5$ | 3.5 3.2 0.9 $\times 10^6$ | 9.6 | 0.06 |
| N845Q  | 7.1 0.1 0.14 $\times 10^7$ | 25.2 1.6 0.7 $\times 10^7$ | 44.3 | 0.014 |
| Q649A  | 6.0 0.1 0.2 $\times 10^5$ | 25.2 1.6 0.7 $\times 10^7$ | 25.3 | 0.023 |
| Q649N  | 8.0 0.1 0.4 $\times 10^5$ | 50.5 0.8 0.16 $\times 10^7$ | 31.3 | 0.019 |
| R668A  | 9.5 0.008 0.8 $\times 10^3$ | 14.1 | 0.042 |
| H811A  | 5.3 0.03 0.6 $\times 10^4$ | 15.8 | 0.040 |
| H811V  | 6.1 0.03 0.5 $\times 10^4$ | 14.2 | 0.040 |
| Q677A  | 72 0.2 0.3 $\times 10^4$ | 41.6 | 0.021 |
| Q677N  | 41 0.04 0.1 $\times 10^3$ | 35.4 | 0.017 |
the differences in the binding modes of WT and mutants of H-bonding track residues was the determination of the effect of correct (complementary) and incorrect (noncomplementary) nucleotides on the binding affinity of the wild-type and mutant enzymes for template-primer. It has been shown before that the presence of correct incoming nucleotide enhances the ability of wild-type KF to bind the template-primer (19, 25, 26). In contrast, the presence of incorrect incoming nucleotide significantly decreases the binding affinity of wild-type KF for template-primer (25, 26). We utilized this property to find out if the mutant enzymes interacted differently with template-primer in the presence or absence of dNTPs. The presence of substrate or nonsubstrate dNTPs (panels C–F) in the presence of correct incoming dNTP, the binding affinity of KF for both 21/12 + ddc and 33/20 + ddc template-primer is increased by ~3–4-fold. In contrast, there is no significant change in the binding affinity of either R668A or Q849N for both template-primer in the presence of substrate or nonsubstrate dNTPs (panels C–F).

In the presence of three incorrect dNTPs, there was a small increase in $K_{D_{DNA}}$ of R668A (from 14 to 24 nM), whereas Q849N showed little change in $K_{D_{DNA}}$ (31 to 27 nM) under these conditions. Similar results were obtained with all the mutants of the H-bonding track residues (data not shown). It is therefore clear that the presence or absence of dNTP minimally affects the DNA binding properties of H-bonding mutants of the Klenow fragment suggesting that the template-primer binding seen at the supershift position may not be related to the polymerase mode of binding but may represent an altered mode of binding with significant decrease in the binding affinity.

Assessment of the Stable Ternary Complex Formation by the Wild-type and Mutant Enzymes—To assess the ability of mutant enzymes to form a stable ternary complex (i.e. enzyme-template-dNTP complex), we used the stable ternary complex formation assay initially developed by Tong et al. (20). This assay has been successfully used to demonstrate the ability or inability of mutant enzymes to form a stable ternary complex, as judged by the differences in the mobility of the complexes formed with each enzyme concentration. The data were fit to a hyperbolic function by nonlinear regression for the determination of $K_{D_{DNA}}$.

**Fig. 2.** The template-primer binding affinity of the wild-type KF with 33/20 + ddC and 21/12 + ddC. This figure shows the template-primer binding affinity of wild-type Klenow fragment with two template-primers containing different lengths in the double stranded region. Panel A depicts the binding affinity of KF to 33/20 + ddC (containing 21-base pair duplex), whereas panel B shows the binding profile of 21/12 + ddC (containing 13-base pair duplex). The lanes marked “input” represent the total amount of radiolabeled template-primer present in the individual reaction. The enzyme concentrations used differ for the two template-primers as the one containing shorter duplex (21/12 + ddC) has greater affinity than the one with longer double stranded DNA (33/20 + ddC). Thus, the enzyme concentration used in panel A ranges between 0.3 and 20 nM, whereas that used in panel B ranges between 0.04 and 1.24 nM. The template-primer concentration used was ~100 pM. In both panels A and B, the last two lanes depict the pattern of migration of the enzyme-TP complex with saturating concentrations of the enzyme (75 and 150 nM). In contrast, no supershifted species is formed when 21/12 + ddc is used at any enzyme concentration (panel B). In panels C and D, the amount of enzyme-TP complex has been plotted as a function of increasing concentrations of the wild-type KF. The percent enzyme-TP complexes were calculated by subtracting the free template-primer in the individual reaction from the total input value. The data were fit to a hyperbolic function by nonlinear regression for the determination of $K_{D_{DNA}}$.
FIG. 3. The template-primer binding affinity and its mode of binding with H-bonding track mutant enzymes. The affinity of H-bonding track mutant enzymes (represented by R668A and Q849N) for template-primer was determined in a manner similar to that used for wild-type enzyme (Fig. 2). Whereas the template-primer concentration was maintained at ~100 pm, much higher quantities of mutant enzymes were required, because of their decreased and variable affinity for template-primer. The panels A and B show the binding of 33/20 + ddC and 21/12 + ddC template-primer, respectively, to the indicated enzymes. The concentrations of both R668A and Q849N mutant enzymes varied between 4.6 and 153.6 nM. The quantitation of enzyme-TP complex formation with the mutant enzymes was carried out based on the quantity of the uncomplexed template-primer, because no clear-cut band migrating at the enzyme-TP position can be detected. For example, in panel A, the binding of R668A to 33/20 + ddC shows a progressive decrease in the free template-primer with increasing concentration of R668A. However, in panel B, with the same mutant enzyme and 21/12 + ddC template-primer, a progressive decrease in free template-primer is accompanied by a "smudge" above the free template-primer position. We have considered the smudge as a part of enzyme-TP complex, although the strength and stability of this binary complex is certainly compromised. In all cases, the quantitation of the area corresponding to free TP position has been used to draw the curves shown in panel C. Because of the extremely low affinity of Q849N for 21/12 + ddC template-primer, the binding curve could not be obtained. The $K_{d, DNA}$ was estimated by fitting the data to a hyperbolic function by nonlinear regression. To demonstrate that the mutants bind the long duplex containing template-primer only in the supershifted mode, two wild-type enzyme concentrations (lanes 8 and 9) were included along with R668A. Three concentrations of wild-type (lanes 8–10) enzyme were included to show the same phenomenon with Q849N. The concentrations of wild-type enzyme according to lanes 8 and 9 of the left two figures were 4.8 and 75 nM, respectively. The concentrations of wild-type enzyme for lanes 8–10 was 3.2, 7.2, and 25 nM, respectively. The comparison of the migration of enzyme-TP complexes with mutant enzymes and wild-type shows that the mutant enzymes bind 33/20 + ddC only in supershifted form. This observation was noted for all mutants of the H-bonding track.

The results of this investigation for the wild-type and three mutant enzymes are shown in Fig. 5. The lane marked input represents the mobility of the template-primer alone. The next four consecutive lanes marked "enzyme + TP," "enzyme + TP + trap," "enzyme + TP + dNTP," and "enzyme + TP + dNTP + trap" show: (i) the binding of template-primer alone, (ii) the effect of the addition of ~500-fold nonradioactive template-primer (trap), (iii) the putative ternary complex in the presence of complementary incoming dNTP, and (iv) the susceptibility of the ternary complex to trap, respectively. It is clear from the figure that enzyme-TP complex between the wild-type (or mutant species) is readily competed out by the addition of the trap template-primer (see lanes marked Enzyme + TP + Trap and compare with Enzyme + TP lanes). However, the complex between wild-type KF and template-primer + dNTP could not be competed out by the same (trap) template-primer (see lane marked Enzyme + TP + dNTP + Trap for WT enzyme). For the mutant enzymes, the enzyme + TP complex in the presence of dNTP was competed out by the nonradioactive template-primer trap suggesting that the addition of dNTP to the enzyme-TP complex failed to form a stable ternary complex. A small amount (~5% of wild-type) of stable ternary complex was noted for H881A. This is consistent with some catalytic activity seen with His881 mutants of KF.

UV-mediated Cross-linking of Enzyme-TP and the Addition of Complementary dNTP in Situ by E-TP Complex—The data presented above suggest that the mutant enzymes of Asn845, Gln849, Arg668, His881, and Gln677 are generally defective in template-primer binding and that the little binding that occurs is probably in the nonpolymerase mode. To further assess if the small extent of binding of template-primer to various mutant enzymes is in the polymerase mode, we used UV-mediated photocross-linking of enzyme to template-primer and examined the ability of individual enzyme-TP complexes for their ability to incorporate the first complementary nucleotide. It has been shown previously that the Klenow fragment of E. coli DNA polymerase I, covalently cross-linked to template-primer, is capable of adding one nucleotide onto the photocross-linked template-primer (18, 22). When covalently cross-linked to the template-primer, translocation ability of the enzyme along the template-primer is compromised. Under these conditions, the nucleotidyl transfer reaction is restricted to the incorporation of the first incoming nucleotide, provided that the 3'-OH of
the primer is correctly positioned in the complex for an in-line attack on the \(\beta\)-phosphate of incoming dNTP. In general, a larger quantity of mutant protein was required to obtain approximately equal extent of cross-linking as that of wild-type KF. To determine the efficiency of enzyme-template-primer cross-linking by mutants compared with wild-type KF, the radioactive template-primer were used to produce enzyme-TP cross-link adducts. The quantitation of these adducts with varying concentration of individual mutant enzymes (data not shown) provided the guidance for the generation of approximately equal quantity of enzyme-TP complexes for all mutant enzymes. For the dNTP addition reaction, all mutant enzymes as well as the wild-type KF were covalently cross-linked to nonradioactive 32/19-mer template-primer and addition of [\(\alpha\)-\(\beta\)P]dCTP (the first incoming substrate dNTP for this template-primer) was assessed. The \textit{in situ} catalytic addition of dCTP was performed in 1 M NaCl to ensure that the addition reaction would be restricted only to the cross-linked enzyme-template-primer species. The results are shown in Fig. 6. A comparison of the intensity of radiolabeled band (representing the addition of radiolabeled nucleotide) shows that none of the mutant enzymes were able to incorporate dNTP onto the cross-linked template-primer to any significant extent (Fig. 6). Compared with the wild-type enzyme, less than 2% incorporation was seen with N845A, R668A, and H881A mutant enzymes. No visible incorporation was noted for Q849A and Q677A enzymes. These results strongly suggest that the 3'-OH of the template-primer cross-linked or bound to mutant proteins was not properly placed at the active site for catalysis to occur.

**DISCUSSION**

The reaction mechanism of DNA polymerases has been well studied (27–29). In addition, the crystal structures of several template-primer and/or dNTP-bound DNA polymerases have provided significant insight into the understanding of the DNA replication mechanisms offered by the nucleic acid polymerases (4–7, 11, 24, 30, 31). The first step in the DNA replication kinetic pathway is the binding of enzyme with TP. The com-
KF equivalents of Asn845, Gln 849, Arg 668, His 881, and Gln 677 of five invariant amino acid residues interacting via H-bonding involving the side chains of several amino acid residues in its template-primer also induces new intramolecular network(s) addition to these large conformational changes, the binding of cleft, and (ii) the transition of J-helix to a coiled structure. In contrast, except for the H881A enzyme, which forms −5% stable ternary complex compared with the wild-type KF, all other mutant enzymes failed to form stable ternary complexes. Note that the addition of excess nonradiolabeled template-primer readily competes out the bound template-primer in the binary as well as ternary complexes for all mutant enzymes (lanes marked enzyme + TP + Trap). The concentration of wild-type, H881A, N845A, and Q677A enzymes was 1.2, 18.3, 150, and 75 nm, respectively.

Fig. 5. Ability of wild-type KF and H-bonding track mutants to form a stable ternary complex. The ability of the wild-type and three mutant enzymes, H881A, N845A, and Q677A, to form stable ternary complexes in the presence of cognate incoming dNTP was assessed following the protocol described under “Experimental Procedures.” The sensitivity of radiolabeled template-primer (33/20 + ddC) in the desired enzyme-TP complexes to the addition of 500-fold excess unlabeled DNA, (which completely dissociates the radiolabeled DNA) provided an indication of ternary complex formation. The radiolabeled template-primer in the complex formed by the wild-type enzyme in the presence of cognate nucleotide (lane labeled Enzyme + TP + dNTP) is not competed out by addition of excess unlabeled DNA (shown in lane Enzyme + TP + dNTP + Trap). In contrast, except for the H881A enzyme, which forms −5% stable ternary complex compared with the wild-type KF, all other mutant enzymes failed to form stable ternary complexes.

Fig. 6. In situ incorporation of dNTP onto enzyme-TP covalent complexes of H-bonding track mutant enzymes. The wild-type (30 pmol) and different amounts of Asn845, Gln677, Arg668, His881, and Gln77 mutant proteins were cross-linked with unlabeled 32/19-mer as labeled as (Bst) DNA polymerases in apo (Protein Data Bank mophilus (Fig. 1). The distance between the Cα atoms of residues at either end of the straight line (Gln677 at one end and Asn845 at the other) is −18 Å and hence we have described it as an −18-Å long H-bonding track. The binding of the substrate dNTP to enzyme-TP complex causes a major conformational change in the fingers domain of pol I (5,11). The process of enzyme-TP-dNTP complex formation is popularly referred to as “fingers closing” or “prepolymerase” ternary complex formation (32). Interestingly, the straight line H-bonding network of interactions remains unaltered in the ternary complex. To understand the function of this interaction network, we employed site-directed mutagenesis to generate mutant derivatives of all five residues and biochemically characterized individual mutant enzymes for its polymerase activity, steady-state kinetic properties, template-primer binding affinity, and the ability to incorporate dNTP substrate onto the primer terminus in situ.

Architecture of the H-binding Track—In the crystal structure of KF and analogous DNA polymerases, the Arg668 equivalent residue is at the center of the H-bonding track and is located at β8. This residue is a part of the highly conserved TGR motif found in the polymerase I family of enzymes (33). In the template-primer bound crystal structures of KlenTaq, the Arg668 equivalent residue (Arg573) is flanked by Gln754 (Gln849 in KF), Gln849 and His786 (His881 in KF). The two distal residues are the equivalents of Asn845 and Gln677 (Fig. 1). In the binary complex of KlenTaq, the amino acid residue equivalent to Arg668 of KF forms a hydrogen bond with Gln849 (3.44 Å), which in turn forms a hydrogen bond with Asn845 (3.69 Å). On the other side, Arg668 has van der Waals interactions with His881. The distance between two of the nearest atoms of Arg668 (Ng) and His881 (C82) is 3.38 Å. There also exists a possibility of a H-bond between atom Ng2 of Arg668 and Nδ1 of His881. However, the distance between the two H-bond forming atoms (4.16 Å) is slightly unfavorable. Furthermore, His881 forms a hydrogen bond with Gln677 (3.33 Å). There are also two intermolecular hydrogen bonds mediated by Arg668 in the enzyme-TP binary complex (11). One is with the primer base (2.39 Å) and the other is with template nucleotide (3.8 Å) base. Similarly,
Gln$^{849}$ and Asn$^{845}$ form hydrogen bonds with template strand. Gln$^{849}$ hydrogen bonds with the base moiety of the template nucleotide (3.15 Å) paired with the base at the primer terminus and Asn$^{845}$ forms a hydrogen bond with the sugar oxygen of the same template base. Amino acid residue Gln$^{677}$ is positioned between the 3rd and 4th base pairs from the primer terminus and has potential to form a hydrogen bond with the base moiety of 3rd primer base in the double strand region of the template-primer. In the binary complex crystal structures of KlenTaq (11) and Bst polymerase (24), Gln$^{677}$ equivalent residues do not form a hydrogen bond through their side chains. However, the possibility of a water-mediated hydrogen bond with the base moieties of the 3rd and 4th base pairs in the double stranded region from the primer end cannot be ruled out as the side chain of Gln$^{677}$ equivalent residues is positioned within the minor groove of the template-primer. A possibility of hydrogen bond between Gln$^{677}$ and base moiety of the primer strand can also be extrapolated from the ternary complex crystal structure of T7 DNA polymerase. Gln$^{430}$ in the J-helix region of T7 DNA polymerase, which can be considered equivalent to Gln$^{677}$ of KF, forms a hydrogen bond with base moiety of the 3rd nucleotide from the primer end (5).

From the detailed analysis of the properties of mutant species of various residues, it is clear that the substitution of the H-bonding side chain in any of the five residues disrupts the track; however, the degree of disturbance is probably different, because the catalytic activity of the individual mutant protein shows variation (Table I). We shall first discuss the participation of individual members and its mutant derivatives deduced from the structural and the experimental results.

**Properties and Environment of Asn$^{845}$ and Gln$^{849}$** —In KF, the sequence NAPMQG$^{850}$ contains two highly conserved uncharged polar residues common to all members of the polymerase I family of enzymes. These are Asn$^{845}$ and Gln$^{849}$. Previous studies have suggested that both Asn$^{845}$ and Gln$^{849}$ play some role in catalysis of the polymerase reaction (13, 14). Our kinetic characterization shows that the $K_{m, \text{ANTP}}$ for N845A and N845Q did not change significantly. However, a significant change was noted in $k_{\text{cat}}$ values for the two Asn$^{845}$ mutant enzyme species. It is rather curious that the N845Q mutant enzyme was significantly more affected compared with N845A. The loss of catalytic activity of the Gln$^{849}$ mutant derivatives (Q849A and Q849N) was more severe suggesting that the side chain of Gln$^{849}$ participates directly or indirectly in the catalytic process. Previously, Polesky et al. (13) have suggested that Gln$^{849}$ interacts with the DNA primer terminus and has some role in dNTP turnover, because Q849A and Q849E exhibited deficiency in the turnover of α-thio-dNTPs. The latter observation also indicates function of Gln$^{849}$ at the chemical step of the reaction. However, the crystal structures of KlenTaq (Klenow fragment of T. aquaticus DNA polymerase I) in three different forms (binary, open ternary, and closed ternary complexes) (11) as well as the crystal structure of T7 DNA polymerase (5) show that the Gln$^{849}$ equivalent glutamine interacts with template nucleotides and it does not have any interaction with dNTP. Therefore, the suggested participation of Gln$^{849}$ in the chemical step (13) is most likely a consequence of a loss in the template-primer binding affinity and/or altered position of 3' OH relative to the active site.

**Properties and Environment of Arg$^{668}$** —Extensive biochemical characterization of R668A has been reported previously by Polesky et al. (13). The results showed a moderate change in $K_{m, \text{ANTP}}$ (−2-fold). Significant changes in $K_{D, \text{DNA}}$ (−20-fold) and $k_{\text{cat}}$ (−400-fold) were noted for the R668A mutant enzyme. Our estimations of $K_{m, \text{ANTP}}, K_{D, \text{DNA}}$, and $k_{\text{cat}}$ for the same mutant enzyme show similar changes. In fact, the $K_{D, \text{DNA}}$ was increased by −25- and −160-fold with 33/20 + dC and 21/12 + ddC, respectively. The $k_{\text{cat}}$ was reduced by −350-fold with dAp-dTAP as template-primer. These data suggest that the defect in the catalytic ability of the R668A mutant was predominately because of the defect in the binding of the template-primer. The interaction of Arg$^{668}$ seen in the crystal structure complexes with template-primer supports the decreased affinity of the R668A mutant enzyme. The side chain position of Arg$^{668}$ in ternary complex crystal structures is favorable for a hydrogen bond with the sugar moiety of dNTP. However, the major participants in the binding of dNTP to enzyme-TP complex appear to be the members of the O-helix and metal ligated Asp$^{705}$ and Asp$^{762}$. Therefore, the weak hydrogen bond between Arg$^{668}$ and the sugar oxygen of dNTP may not be contributing significantly to binding and stabilization of dNTP at the active site.

The role of an amino acid residue at the chemical step of nucleotide incorporation by DNA polymerases has been judged by the difference in utilization of normal versus α-thio-substituted dNTPs (32). This is called an elemental effect. R668A mutant protein has been shown to utilize dNTPαS about 10-fold less efficiently than the wild-type enzyme, suggesting the involvement of Arg$^{668}$ at the chemical step. Arg$^{668}$ has been shown to form the ion pairs with Glu$^{710}$ which has been proposed to ligate a metal ion coordinating with the 3’-OH of dNTP (34). Thus the negative effect of R668A on the chemical step may be proposed because of the loss of its reactivity with Glu$^{710}$, and the elemental effect seen for the R668A mutant may be the reflection of some indirect effect mediated through Glu$^{710}$.

**Properties and Environment of His$^{881}$** —The biochemical characterization of His$^{881}$ shows that the H881A mutant is ~30% as active as WT. There is no significant change in $K_{m, \text{ANTP}}$, whereas a 6-66-fold decrease in $k_{\text{cat}}$ with different template-primers was noted, suggesting the requirement of His$^{881}$ for optimal catalysis by KF. The location of His$^{881}$ is on a β-turn between β12 and β13 in the palm subdomain. It is highly conserved among pol I type DNA polymerases. In the crystal structure of T7 and TaqDNA polymerases, the equivalent residues interact with the sugar moiety of the primer terminus nucleotide (5, 11). Therefore, this residue has been proposed to position the primer terminus for catalytic activity (5). Comparison of theapo and binary complex of TaqDNA polymerase crystal structures shows that the turn between β12 and β13 undergoes a “springboard-like” conformational change upon DNA binding, which does not change further upon ternary complex formation. The Ca distance between two His$^{881}$ conformations (in apo-KlenTaq and template-bound KlenTaq) is ~3 Å. Superposition of apo onto the binary complex shows that if such a sharp springboard-like conformational change of the turn between β12 and β13 were not to occur, His$^{881}$ would interfere with the sugar moiety of the primer terminus nucleotide. Analyses of these crystal structures suggest that two additional residues probably contribute to induce a springboard-like conformational change. These residues are Gln$^{777}$ and Gln$^{779}$. In the binary and/or ternary complex, His$^{881}$ side chain forms a hydrogen bond with Gln$^{777}$, whereas the main chain carbonyl forms a hydrogen bond with Gln$^{779}$. In addition, Gln$^{779}$ forms a hydrogen bond with Gln$^{777}$. It appears that the triangular H-bond arrangement at this site may be responsible for the conformational change in the β-turn, which can then accommodate the template-primer terminus at a proper position.

**Properties and Environment of Gln$^{677}$** —Gln$^{677}$ belongs to the J-helix in pol I-type DNA polymerases. This helix is formed by five amino acid residues flanked by proline on either ends. The sequence of this region is highly conserved. The J-helix under-
goes a conformational change (from helix to coil) upon TP binding (5, 11). However, the helical structure is maintained when the binding of the template-primer occurs in the 3'-5' exonuclease mode (9). We have previously shown that helix to coil transition (or vice versa) of the J-helix plays a crucial role in the switching of the template-primer from the polymerase to the exonuclease site, as judged from the properties of P680G (35). Upon the binding of template-primer to the enzyme in the polymerase mode, repositioning of the side chain of Gln^{677} has also been noted. This in turn allows Gln^{677} to make contacts with His^{881} and Gln^{879}. The mutant Q677A enzyme is an inactive enzyme (35, 36). Our activity and steady-state kinetic data also show severe loss in the polymerase activity of both nonhomologous Q677A and homologous Q677N mutant enzymes (Table II). In fact, the polymerase activity of the P680G mutant of KF was also reduced to the same extent as that seen for Gln^{677} mutant enzymes (35). Therefore, it is possible that the reduced activity of P680G was a reflection of the activity of the enzyme because of the altered position of Gln^{677}. A change in the position of Gln^{677} would result in the destabilization of the H-bonding track arrangement, which in turn would not permit the binding of enzyme to template-primer. Whereas the polymerase activity of the P680G mutant enzyme was severely reduced, the mutant enzyme was extremely efficient in the 3'-5' proofreading activity (35). We have noted a similar increase in the efficiency of 3'-5' exonuclease activity with the Q677A mutant species.2

Thus, the comparison between the activity and steady-state kinetic constants of the wild-type and mutant enzymes (Table II) suggests that all of the H-bonding track residues participate in the catalytic function of Klenow fragment of \textit{E. coli} DNA polymerase I. The major participation of H-bonding track residues is in the process of the template-primer binding, as judged by the significant decrease in $K_{D_{\text{DNA}}}$ (Table II). These observations are consistent with the crystal structure data of the binary and ternary complexes of KlenTaq, which show that these residues interact with the template-primer (11). The $K_{D_{\text{DNA}}}$ for some of the mutant enzymes studied here was also determined by others (13). They noted that the N845A mutant showed no change in template-primer binding affinity compared with the wild-type KF. However, the other mutant of Asn^{845} (N845D) had a 15-fold decrease in template-primer binding affinity. The $K_{D_{\text{DNA}}}$ for H881A was altered by only 2.5-fold, whereas this value for Q849A and R668A mutants was reported to be 10- and 17-fold greater, respectively (13). The change in $K_{D_{\text{DNA}}}$ for N845A, Q849A, R668A, H881A, and Q677A with 33/20 + ddC, in our study, is 16-, 43-, 22-, 25-, and 70-fold, respectively. Thus, the $K_{D_{\text{DNA}}}$ values determined here are significantly different from those reported by Polesky \textit{et al.} (13). The differences in $K_{D_{\text{DNA}}}$ values may be attributed to two different methods and the template-primers used in two studies. In fact, we have noted that $K_{D_{\text{DNA}}}$ values vary significantly, based upon the method of determination as well as the concentration of template-primer (19).

The structural data on enzyme-DNA binary complexes of KlenTaq and \textit{B. stearothermophilus} Klenow fragment and the ternary complexes of KlenTaq and T7 DNA polymerase I show that 8–10 base pairs of the double stranded DNA are occluded by these enzyme proteins. The template-primers in these crystal structure complexes have between 30 and 45 interactions. A majority of these interactions are in the double stranded region of the template-primer. Despite the large number of interactions, the interactions provided by the H-bonding track residues appear to be the most significant for retaining the bound template-primer. The fact that mutation at any one of the five residues produces significant loss in binding affinity for TP further suggests that these residues operate in concert and that the formation of H-bonding track concurrent with TP binding in the active center may provide an appropriate geometrical conformation for landing of the correct dNTP substrate.

**Template-primer Binding by the Mutant Species of H-bonding Track Residues**—Another interesting feature of all the mutant enzymes that we noted is that they are not only defective in template-primer binding, but quality of their binding is also different from that seen with the wild-type enzyme. For example, they bind the longer duplex containing template-primer in a supershifted mode (Figs. 2 and 3). It has been noted previously that increasing the concentration of KF results in the spontaneous shift in the retardation pattern of the enzyme-TP complex species from a regular to supershifted position (15, 25, 37–39). It has been suggested that the supershifted species of enzyme-TP complex corresponds to the binding of two or more enzymes to the same template-primer. The DNA footprinting and crystallographic data on KlenTaq, T7 DNA polymerase, and \textit{B. stearothermophilus} have implicated that the Klenow fragment occludes 8–10 base pairs (5, 11, 24) of the template-primer. Furthermore, these mutant enzymes appear unable to stably bind the template-primer containing a shorter (13 base pair) double stranded region (Figs. 3 and 4).

These observations suggest that the duplex region in the 33/20 + ddC template-primer, which is not occluded by the enzyme, can support the binding of another KF molecule to produce a supershifted position of migration. A plausible way that the second molecule of KF can bind the unoccupied region of duplex is its binding at the blunt end of the duplex with significantly lower affinity, thus requiring a higher quantity of enzyme protein. Obviously, this situation is not possible for the shorter duplex containing template-primer, for its length of the double stranded region may not be enough to permit the binding of a second molecule. The relatively lower affinity of the wild-type KF for 33/20 + ddC compared with 21/12 + ddC template-primer is therefore likely to be because of the presence of two sites in the former template-primer compared with only one site in the latter. The binding of two (mutant) enzymes to the longer duplex containing template-primer could conceivably occur in an “exit mode” because of their inability to bind at the 3' terminus flanked by a single stranded template overhang at the appropriate orientation and position. This might permit the binding of two mutant enzymes in a nonproductive manner (back-to-back binding), using both termini of the template-primer. The wild-type enzyme, in contrast, would bind the bona fide 3' terminus with high affinity and the nonproductive 3' terminus (of the same template-primer) with low affinity. The capability of Taq polymerase to bind a duplex DNA in the latter mode is well documented by Eom \textit{et al.} (31). In the crystal structure, the authors have shown that the 3'-OH of the duplex is bound close to the active site and that this type of binding has been called the exit mode binding.

The observations on the effect of dNTP addition on the stability of the enzyme-TP binary complex also supports the notion that the binding of the template-primer to mutant enzymes is of a different nature than that seen with wild-type. For example, the addition of the next incoming dNTP complementary to the template nucleotide increases the affinity of the wild-type enzyme for template-primer, as judged by a ~3–4-fold decrease in the $K_{D_{\text{DNA}}}$ In contrast, the addition of noncomplementary dNTPs significantly reduces the binding affinity of the wild-type enzyme for the template-primer (Fig. 4, panels A and B). All of the mutant enzyme-TP complexes re-

\footnote{K. Singh and M. J. Modak, unpublished data.}
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Functional Motifs of pol I—The compilation and alignment of the amino acid sequence of many DNA polymerases have shown that certain motifs (motifs A–E) are conserved in different members of the DNA polymerase family (1–3, 40, 41). Two of these motifs (A and C) are conserved across all known DNA polymerases. In the pol I family of DNA polymerases, five sets of conserved amino acid sequences were identified (1). These conserved sequences were numbered as regions 1–5. The conserved regions 3–5 were designated as motifs A–C, respectively (1). In the pol I class of enzymes, the catalytically essential aspartates (Asp705 and Asp882) are present in motifs A and C, whereas motif B contains 4 catalytically important residues of the O-helix. Region 1 is part of the thumb subdomain and appears to be somewhat isolated from other motifs. A part of region 2 contains the conserved sequence TGR; the Arg residue in this sequence is Arg668 of KF. Interestingly, this motif was later specified as the (T/D)XXG motif that occurs in T7 RNA polymerase (33). In addition to these identified conserved regions, it appears that three additional conserved motifs could be added to this list based on their functional importance in the pol I family. These motifs are represented by (i) the J-helix region, (ii) the hinge region between M and N helices, and (iii) a part of the Q-helix containing the NXXXGQ sequence. We would like to suggest that the sequence PXXQXXG (corresponding J-helix) be defined as J-motif, where P is proline, Q is glutamine, and X is a variable residue. The second motif represented by GDXWH, which was previously recognized as the GDX hinge region in KF and Mycobacterium tuberculosis (15) pol I, may now be called the H-motif. The importance of the third motif represented by the NXXXGQ sequence was also recognized by Loeb and co-workers (42) and they labeled it as region 6. We suggest that because of the functional importance of both Asn and Gln in this sequence, it be called the NQ motif. These three motifs and their conservation in the polymerase I family of enzymes are shown in Fig. 7A. Thus, there are now at least 8 functionally important conserved regions in the pol I family of enzymes. The location of these conserved motifs also provides some insight into their functional importance, as most of these residues are located surrounding the active site in the polymerase domain (Fig. 7C). Using the biochemical data presented here and those reported previously, coupled with structural data on KF and related enzymes, we find that during the catalytic process, all of the conserved motifs interact with each other to effect the optimal binding of substrates leading to efficient chemical reaction. The scheme of their interactions is shown in Fig. 7B. The center of the interaction is at Arg668. During the template-primer binding, the conformational change is accompanied by interactions among motifs TGR, A, C, J, and NQ. This is followed by the binding of dNTP, which induces the conformational change mediated by Asp705 of H-motif (15) such that a new interaction between motifs A and B is established to form a closed pre-polymerase ternary complex (6, 11).

Does H-bonding Track Provide Flexibility to the 3′-5′ Exonuclease Domain—Another unique aspect of pol I-type enzymes is the presence of a 3′-5′ exonuclease domain, which operates at some 30–35 Å from the polymerase active site. This domain is conserved even among the members (e.g., T. aquaticus pol I and M. tuberculosis polymerase I) that do not exhibit catalytic activity through this center. Nevertheless, in the pol I family, the importance of the 3′-5′ exonuclease domain is recognized by its proofreading activity (i.e., exonucleolytic removal of mismatched nucleotide). Of the two models for the proofreading function of pol I, one involves the shuttling of a primer strand containing terminal mismatched nucleotide from the polymerase site to the exonuclease site (43). The second mode suggests a dissociation of enzyme from the template-primer upon mis-incorporation, followed by rebinding of an enzyme to the mismatched template-primer, in the 3′-5′ exonuclease mode (43). Regardless of which mode is operative, the binding of mismatched template-primer to enzyme probably requires part of the same binding track with some alterations, whereby part of the primer strand may be oriented toward the exonuclease site. Based on the crystal structure of the ternary complex of T7 DNA polymerase, Doublie et al. (5) have proposed that such an alteration in template-primer binding contact points may be required in directing the primer terminus to the exonuclease site and that residues equivalent to Arg668 and Gln849 of KF may be involved in this process. Similar shuttling between polymerase and exonuclease sites with similar changes in the contact points have been proposed for RB69 DNA polymerase by Franklin et al. (12). Earlier, we have reported that alteration in the J-helix region (Gln877 is a member of this region) also triggers preferential binding of the primer strand to the exonuclease site (35). The fact that 3 of 5 members of the H-bonding track are also involved in the binding or shifting of template-primer to the exonuclease mode strongly suggests that the 18 Å long H-bonding track may be uniquely found in enzymes that contain both polymerase and exonuclease domains. Furthermore, the binding of primer moiety (containing mismatched terminal nucleotide) could conceivably occur via alteration in the H-bonding track. The RB69 DNA polymerase, a member of the pol α family (40), has both polymerase and 3′-5′ exonuclease activity domains and it also contains an H-bonding track, similar to pol I, which can be readily discerned from its ternary complex crystal structure (Fig. 7D). Conversely, such a track is absent in enzymes lacking the 3′-5′ exonuclease domain, such as HIV-1 reverse transcriptase and mammalian DNA polymerase β. Therefore, it is tempting to speculate that an H-bonding track may have been involved to permit shuttling of primer from the polymerase to the exonuclease site.

In summary, we have identified an important structural feature in the form of an 18 Å long H-bonding track in the pol I family of enzymes. The track is formed by the concerted action of 5 residues upon the binding of template-primer in the active center of pol I. The mutation of any one of the five residues involved in H-bonding abolishes the binding of template-primer, because the formation of the H-bonding track involves...
the interaction among all of its members, as well as with the sugar and base moieties of four terminal base pairs of template-primer. We conclude that the formation of this track not only provides the stability to bound template-primer, but may be responsible for the reported changes in the conformation of DNA from the B to A-like form at the active center. Inherent flexibility in the H-bonding network may readily permit the displacement of the water molecules near the active site to effect the structural transition of bound template-primer. Such a change may be pre-requisite for the positioning of the primer, orientation of templating base or single stranded template strand to facilitate the binding of the appropriate dNTP. The presence of the H-bonding track seen in both enzyme-TP and enzyme-TP-dNTP ternary complexes strongly supports this contention. The stabilization of the template-primer via the H-bonding track interactions is also likely to permit easy sliding...
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during synthetic reaction as opposed to conventional interactions involving backbone phosphate groups of template-primer. A detailed analysis of the role played by individual members of the H-bonding track is currently under investigation.

Acknowledgments—We thank Drs. Jin Jin and Neerja Kaushik for participation in the early phase of this work. We gratefully acknowledge the editorial assistance of Dr. Herald Calvin.

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J. Biol. Chem. 2003, 278:11289-11302.
doi: 10.1074/jbc.M211496200 originally published online January 9, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211496200

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