The J-helix of *Escherichia coli* DNA Polymerase I (Klenow Fragment) Regulates Polymerase and 3′–5′-Exonuclease Functions*

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To assess the functional importance of the J-helix region of *Escherichia coli* DNA polymerase I, we performed site-directed mutagenesis of the following five residues: Asn-675, Gln-677, Asn-678, Ile-679, and Pro-680. Of these, the Q677A mutant is polymerase-defective with no change in its exonuclease activity. In contrast, the N678A mutant has unchanged polymerase activity but shows increased mismatch-directed exonuclease activity. Interestingly, mutation of Pro-680 has a Q677A-like effect on polymerase activity and an N678A-like effect on the exonuclease activity. Mutation of Pro-680 to Gly or Gln results in a 10–30-fold reduction in $k_{\text{cat}}$ on homopolymeric template-primer, with no significant change in relative DNA binding affinity or $K_m$.

The mutants P680G and P680Q also showed a nearly complete loss in the processive mode of DNA synthesis. And the N678A mutant has unchanged polymerase activity but no change in its exonuclease activity. In contrast, the Q677A mutant is polymerase-defective with no change in its exonuclease activity. The mutants P680G and P680Q also showed a nearly complete loss in the processive mode of DNA synthesis. And the N678A mutant has unchanged polymerase activity but no change in its exonuclease activity. In contrast, the Q677A mutant is polymerase-defective with no change in its exonuclease activity.

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Recent resolution of T7 and KlenTaq DNA polymerase (6–8) crystal structure complexes has provided detailed information on the interactions between DNA-dNTP and protein in binary and ternary complexes. In addition, these crystal structures have revealed conformational changes in various enzyme subdomains and DNA that appear to be a prerequisite for the formation of proper active site geometry for dNTP incorporation. A detailed examination of the binary and ternary complex crystal structures of the pol I family of DNA polymerases has revealed sequential conformational changes, relative to the unliganded protein, upon template-primer and dNTP binding, respectively (7–8). Template-primer binding is associated with translational and rotational changes in the thumb subdomain, described as “clamping down” over DNA (3). Subsequently dNTP binding induces movement in the fingers domain (mainly O-helix) by $\pm 41^\circ$, which in turn forms the “closed” ternary complex.

In addition to the conformational change in the thumb subdomain upon template-primer binding at the polymerase site, a smaller conformational change is observed in the J-helix region (7–8). A shift of approximately 13° away from the active site (our observation) as well as a helix-to-coil transition of this region are noted upon binary complex formation (Fig. 1). Superposition of apo-KlenTaq on the template-primer-bound enzyme structure reveals that this conformational change is necessary to accommodate duplex DNA at the active site. Moreover, van der Waals and H-bond interactions between the J-helix main chain peptide backbone and the DNA sugar-phosphate backbone occur mainly within the minor groove, three bases upstream of the 3′ terminus (7–8).

In addition to the contacts observed for the J-helix with DNA at the polymerase domain, examination of the “editing mode” DNA-bound crystal structure of the KP (3, 9) shows that the J-helix residues maintain an $\alpha$-helical conformation and that two residues of the J-helix, Asn-675 and Asn-678, form H-bond contacts with the phosphate oxygen atoms of the primer strand. These observations led us to investigate the role of the individual members of J-helix in the polymerase function and, more specifically, their role in integrating the polymerase and exonuclease activities of Klenow fragment. It had been proposed (2) that a coordination of polymerase and proofreading activities is facilitated through interactions of a region in the enzyme protein with the minor groove of DNA at the polymerase-active site. This region has been described as a “reading head” that could detect DNA lesions due to mispairing, presumably as a result of perturbations in the minor groove geometry (2). In order to determine the role of the J-helix residues in the coordination of polymerase and exonuclease activities, we performed site-directed mutagenesis of five resi-

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1 The abbreviations used are: KP, Klenow fragment; DTT, dithiothreitol; BSA, bovine serum albumin; TP, template-primer; WT, wild type; PAGE, polyacrylamide gel electrophoresis; E-TP, enzyme template-primer complex; ssDNA, single-stranded DNA.
dues of the J-helix region. The work presented here provides evidence identifying the J-helix as a candidate for such a function. We show that mutations in this region result in enzymes with reduced polymerase activity. Moreover, we find that the P680G mutant exhibits increased exonuclease activity on the mismatched template-primer. These results are in conjunction with structural observations on apo-KlenTaq, binary complex, and Klenow fragment editing complex suggest that the J-helix coordinates polymerase and 3′-5′-exonuclease activities of the Klenow fragment, probably through its interactions with the DNA minor groove at the polymerase-active site.

**EXPERIMENTAL PROCEDURES**

*Materials—*Mutagen M13 in vitro mutagenesis kit was purchased from Bio-Rad. Sequenase and DNA sequencing reagents were purchased from United States Biochemical Corp. Restriction endonucleases and DNA-modifying enzymes were obtained from Promega or Roche Molecular Biochemicals. High pressure liquid chromatography-purified dNTPs were purchased from Roche Molecular Biochemicals. ³²P- and ³H-labeled dNTPs were purchased from NEN Life Science Products. Bio-Rex 70 cation exchange resin was from Bio-Rad. Polyclonal antibodies were from Sigma, and streptomycin sulfate was from Fisher. Synthetic oligonucleotides used in DNA sequencing, mutagenesis, and biochemical assays were synthesized by the Molecular Biology Resource Facility at the University of Medicine and Dentistry-New Jersey, Newark. DNA purification and extraction kits were purchased from Qiagen.

**In Vitro Site-directed Mutagenesis—**The M13mp19 template carrying a 1.2-kilobase pair SacI and HindIII insert of Klenow fragment-coding sequence of *E. coli* pol I gene was used for site-directed mutagenesis (10, 11). Synthetic oligonucleotides carrying the desired substitutions for mutations were used in the Bio-Rad Mutagen-M13 in vitro mutagenesis kit in accordance with the manufacturer’s protocol to generate the mutants. After confirming the mutation by DNA sequencing (12), the 1.2-kilobase pair mutated SacI and Hind III fragment was subcloned into pET-3a-K plasmid containing the coding sequence for Klenow fragment (11). Mutant plasmids were transformed into the maintenance strain HB101 and expressed in *E. coli* BL21 (DE3).

**Expression and Purification of the WT and Mutant Derivatives of Klenow Fragment—**Induction and purification of the mutants and WT enzymes were carried out as described earlier (11, 13). Briefly, the cells were grown at 37°C to an *A*₅₉₅ of 0.3 followed by induction of the protein by adding 200 μM isopropyl-1-thio-β-D-galactopyranoside. After 5 h of growth at 30°C, the cells were harvested by centrifugation at 5,000 × *g*. The pellet was washed with a buffer containing 50 mM Tris-HCl, pH 7.0, 1 mM DTT, 10 mM EDTA, and 250 mM NaCl and recentrifuged. Purification of the protein was performed as follows. Cell lysis was carried out on ice for 1 h in a buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, and 2 mg/ml lysozyme. The lysate was sonicated briefly to reduce viscosity and then centrifuged at 10,000 × *g* for 1 h. The protein-DNA complexes in the crude extract were precipitated with 0.2% polyethyleneimine, and the insoluble fraction was extracted twice with approximately 4 volumes of 0.1 M NaCl in 10 mM phosphate buffer, pH 7.0, 1 mM DTT. The pooled extracts were fractionated by the addition of ammonium sulfate, and the enzyme was purified with Bio-Rex 70 column chromatography as described earlier (13). Protein concentrations were determined by the Bradford colorimetric assay (14) and confirmed visually by the staining intensity of the protein bands against known concentrations of BSA standard on SDS-polyacrylamide gels (15). The thermolysin sensitivity pattern of the mutant proteins was analyzed by Polesky et al. (16), in order to ensure that no changes in the folding pattern of the enzyme protein (due to mutation) had occurred.

**Specific Activity Determination—**Enzymatic activity was determined at 37°C for 10 min on homopolymeric and heteropolymeric template-primers. The reaction was carried out in a final volume of 100 μl containing 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol (DTT), 0.01% BSA, 500 μM each of the desired template-primer, 5 mM MgCl₂, and 50 μM dNTP (1 μCi/assay) corresponding to the homopolymeric template-primer. Reactions with the heteropolymeric template-primer contained all four dNTPs at a concentration of 50 μM each, with two of them being radiolabeled. Enzyme concentrations ranged from 3 to 30 nM. Reactions were initiated by the addition of MgCl₂ and quenched with 5% ice-cold trichloroacetic acid containing 10 mM PF₃, at desired time intervals. The precipitated material was collected on Whatman...
glass fiber filters and dried, and radioactivity was determined by scintillation counting (11).

**Determination of Relative Template-Primer Affinity for WT and Mutant Klenow Fragments**—Affinity of J-helix mutant enzymes for a 37-mer self-annealing template-primer was determined by cross-linking (11) as described below. DNA binding assays with a single mutant and WT KF were carried out side-by-side in a total volume of 50 μl containing 5 nM 5'-32P-labeled hairpin 37-mer DNA (50,000 cpm/reaction), 50 mM Tris-Bl, pH 8.0, 1 mM DTT, 2 mM MgCl2, and from 1 to 3125 nM Klenow fragments. The samples were incubated on ice for 15 min followed by UV irradiation at 254 nm in a Spectrolinker (Spectronic Corp.) for an energy of 0.35 J/cm2. The cross-linked E-TP species were resolved on an 8% SDS-polyacrylamide gel, visualized by scanning the gel on a PhosphorImager, and the extent of cross-linking quantitated using ImageQuant software. The intensity of the covalent E-TP complex was plotted as a function of enzyme concentration, using GraphPAD PRISM software (GraphPAD Software, Inc.). Assuming that the maximum band intensity of the cross-linked kinetic 37-mer complex represents saturation of the DNA with enzyme, then the concentration of KF required to give one-half maximum intensity reflects K_f0DNA (see “Results” for details). The relative affinity of J-helix mutants for 37-mer is defined as a dimensionless constant K_rel(wild-type)/K_rel(mutant).

**Steady State Kinetics of Polymerization**—Determination of kinetic constants for dNTP substrates was carried out at 37 °C for 5–20 min on homopolymeric (dA)18 template-primers in the presence of varying amounts of dTTP, dATP, dGTP, and all four dNTP substrates, respectively (11, 18). The reaction mixture contained 50 mM Tris-Bl, pH 7.8, 50 mM KCl, 1 mM DTT, 0.01% BSA, 1–2 μM template-primer, 1 μCi/radioactive [32P]dNTP, and 3–30 nM WT or mutant Klenow fragments. The reaction was initiated by the addition of 2 mM MgCl2. Steady state parameters K_m, V_max, and V_max/K_m were determined from Eadie-Hostee plots. k_cat was determined from the equation, V_max = [E]0 k_cat.

**Single dNTP Turnover by WT and Mutant Klenow Fragment**—An assay designed to limit observations to a single dNTP incorporation for a KP-TTP complex was carried out as follows: 5 nM 5'-32P-labeled 16-mer oligomer, annealed to 46-mer template, was incubated at 23 °C for 5 min with 50 nM WT or mutant KP in a buffer containing 50 mM Tris-Bl, pH 8.0, 1 mM DTT, and 100 μM dTTP, in a final volume of 24 μl. The reactions were initiated with the addition of 12 μM of 6 mM MgCl2, and 6-μl aliquots were withdrawn every 5 s for the WT and those mutants exhibiting WT polymerase activity. Reactions catalyzed by mutants with substantially decreased polymerase activity were quenched at 20, 40, 60, 120, and 300 s by the addition of 4 μl of a solution containing 10 mM Tris-Bl, pH 8.0, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol in formamide. The reactions were resolved on a denaturing uncleaved 16-mer polyacrylamide gel. The extent of the reaction was monitored by conversion of the labeled 16-mer to 17-mer. A control reaction to assess the extent of duplex formed by the annealed oligomers was performed with an identical reaction set as described above except that it was allowed to proceed for 20 min. The KP-TP and relatively long reaction time ensures total conversion of the duplex to product.

**Time Course of TTP Incorporation on Poly(dA) Template**—In order to investigate the rate-limiting step on homopolymeric poly(dA) template-primer by the mutant enzymes, we carried out a time course of dTTP incorporation. In this experiment, the extension of the 5'-32P-labeled (dT)18 annealed to poly(dA) template was monitored by gel analysis of the products. The reactions were carried out at 25 °C in a buffer containing 50 mM Tris-Bl, pH 7.8, 1 mM DTT, 5 mM poly(dA)+dT18 (105 cpm/reaction), 0.01% BSA, 200 μM dTTP, and 5 mM MgCl2. Five-microliter aliquots were withdrawn, and the reaction was quenched at 10-s intervals. The enzyme concentration used for this determination was 5 nM WT Klenow and 5–50 nM mutant enzyme. Products were resolved on a urea-12% polyacrylamide gel and visualized by phosphorimaging. For processivity determination, the assays included 100-fold more unlabeled template-primer concentration.

3'-5' Exonuclease Activity Assay—The exonuclease activity of the WT and mutant Klenow fragment was assessed by determining the extent to which the enzymes could degrade the 5'-labeled single-stranded DNA and double-stranded matched and mismatched template-primer (see Chart I). Reactions were carried out by incubating the WT or mutant Klenow fragment (5 nM) with 5 or 500 nM labeled DNA (50,000 cpm/reaction), in a reaction mixture containing 50 mM Tris-Bl, pH 7.8, 1 mM DTT, and 5 mM MgCl2. The reactions were initiated with the addition of Mg2+ and allowed to proceed for 1–20 min and then quenched by the addition of 4 μl of Sanger’s stop solution. Products were resolved by urea-PAGE and visualized by phosphorimaging. The decrease in band intensity of the primer over a period of time was quantitated using ImageQuant software (Molecular Dynamics, Inc.) and plotted as the fraction of full-length labeled DNA remaining as a function of time. The hydrolysis profile was fit to a single exponential decay, and the rate constant (k_0) was calculated from the curve. We also calculated the overall rate at which the three mismatched 3'-nucleotides of the 26/20-mer were excised to yield the correctly paired 26/17-mer and expressed it as k_mismatch. The steady state rate constant for conversion of mismatched 26/20-mer to the matched 26/17-mer was determined as follows. The accumulation of band intensity corresponding to the 17-mer position was quantitated using ImageQuant and converted to nM/s. The extent of primer remaining at 17-mer position was plotted as a function of time, and the rate constants were calculated directly from the slope of the line.

### RESULTS

**Site-directed Mutagenesis of J-helix Residues and Purification of WT and Mutant Enzymes**—In order to understand the participation of the J-helix residues in DNA synthesis, individual residues of this region were mutated as described under “Experimental Procedures.” The J-helix region (Asn-675 to Pro-680) in pol I contains three polar residues (Asn-675, Gln-677, and Asn-678) capable of functioning as both hydrogen bond donor and acceptor, two hydrophobic residues (Leu-676 and Ile-679), and one proline residue (Pro-680) that lies at the end of the helical region. Strucural data on pol I suggest that of the two hydrophobic residues, only Ile-679 interacts with the primer. Therefore, it was included in our studies that mutant derivatives N675A, Q677A, N678A, I679A, and I679L and a double mutant N675A/N678A were constructed and characterized for polymerase and exonuclease functions. The results with purified mutant proteins showed that, with the exception of Gln-677, side chains of J-helix residues listed above contribute minimally to the polymerase function of KP as judged by the result of activity assays. Among these mutants, only the...
The specific polymerase activity of the WT Klenow fragment and its mutant derivatives was determined with the indicated template-primers as described under “Experimental Procedures.” The values shown are expressed as percent wild type activity. The 100% WT activity value in the presence of Mg\(^{2+}\) as divalent cation on either (dA)\(_{36}\) or (dT)\(_{36}\) was 1.2 × 10\(^6\) units/mg of protein, whereas this value was in the range of 3.5 to 6.4 × 10\(^5\) units/mg of protein on (dC)\(_{60}\) and the heteropolymeric 49/18-mer. One unit is defined as the activity necessary to incorporate 10 nmol of dNMP into acid-insoluble form in 30 min at 37 °C.

### Table I

**Specific DNA polymerase activity of the wild type and mutant Klenow fragment**

| Enzyme | (dA)\(_{18}\)/dT | (dC)\(_{18}\)/dA | (dG)\(_{18}\)/dC |
|--------|-----------------|-----------------|-----------------|
| P680G  | 6               | 20              | 11              |
| P680Q  | 2               | 7               | 11              |
| I679A  | 88              | 102             | 150             |
| I678L  | 77              | 110             | 63              |
| N678A  | 80              | 130             | 100             |
| Q677A  | 1               | 7               | 22              |
| N675A  | 90              | 150             | 64              |

N678A mutation showed some change in the exonuclease activity. Therefore, we included an additional site, Pro-680, that could potentially influence the entire J-helix structure, including the positions of Gln-677 and Asn-678 side chains, for mutagenesis. Two mutant derivatives, Pro → Gly and Pro → Gln, were constructed in both exonuclease" and exonuclease" backgrounds, and detailed biochemical investigation of the properties of these mutants was carried out.

To ensure that the mutant proteins have similar globular folding patterns as the WT, the thermolysin digestion pattern of these mutants was carried out.

In the enzymatic process, the mutation of Gln-677, however, exhibited substantial loss in activity. Thus, of the three amido groups containing functional groups (side chain amide and carbonyl moieties) implying no participation of these residues in the synthetic process. The mutation of Gln-677, however, exhibited substantial loss in activity. Thus, of the three amido groups containing side chains, only one (Gln-677) participates in the polymerase function of KF. Since we have found that Gln-677 also contributes to the H-bonding track present at the catalytic center of KF, the characterization of its mutants together with other members of the H-bond track will be presented elsewhere. The terminal residue of this helix, Pro-680 with its inert side chain, was not expected to play a direct role in the enzyme function(s); however, its mutation to Gly or Gln produced a catalytically compromised enzyme, warranting further characterization of P680G/P680Q.

### Determination of Template-Primer Affinity for WT and Mutant KF

Template-primer binding is the first step in the reaction pathway of enzyme-catalyzed DNA synthesis (17). To determine the effect of mutation of individual J-helix residues on template-primer binding, we developed an assay to measure relative DNA-binding affinities by the UV-mediated cross-linking of the 5'-labeled self-annealing 37-mer template-primer to the enzyme protein. The E-TP cross-linked complexes were formed by incubating 5'-labeled DNA (~5 nM) with increasing concentrations of KF, ranging from 1 to 3125 nM, and the relative extent of individual E-TP complex formation was assessed by covalent cross-linking of complexes by UV irradiation. The cross-linking efficiency is generally low (<1%). However, we have determined that this value is constant over the concentration range employed and does provide concentration-dependent classical saturation curve. Therefore, cross-linking profile provides a convenient and reproducible approach for the determination of relative DNA binding affinity. The cross-linked E-TP complexes were resolved by SDS-PAGE, and radioactive bands corresponding to the complex were quantitated. The band intensities were plotted against enzyme concentrations. The data points were fitted to a hyperbolic curve. From these data, the “relative DNA binding affinity,” defined as the concentration of KF that gives ½B\(_{\text{max}}\) (half-maximal saturation) of TP, was estimated. Results from a typical experiment are shown in Fig. 2. Data presented in Fig. 2A and B, correspond to the WT and P680G mutant, respectively.

Table II gives relative DNA binding affinity values for WT and various J-helix mutant derivatives. The relative DNA binding affinity obtained by our cross-linking experiments is 12 nM for WT, which is in good agreement with the values determined by pre-steady state kinetic methods and gel mobility shift assays (18, 19). Only slight variation in the relative DNA binding affinity (3-fold increase or decrease) was observed for different J-helix mutant enzymes, indicating little or no effect of these substitutions on the template-primer binding affinity.

### Determination of Steady State Kinetic Parameters for WT and Mutant KF

The steady state kinetic parameters obtained for the WT and J-helix mutant polymerases are given in Table III. In these experiments, the WT and mutant enzyme activities were examined with four different template-primers (see “Experimental Procedures”) allowing for k\(_{\text{cat}}\) and k\(_{\text{cat}}\)/k\(_{\text{m}}\) determination of three individual dNTPs with homopolymeric TP and an average k\(_{\text{cat}}\)/k\(_{\text{m}}\) for all four dNTPs on a synthetic 49/18-mer DNA. The mutants with little loss of catalytic activity were not expected to exhibit significant changes in their kinetic parameters and therefore were not included in this analysis. P680G and P680Q showed significant decreases in k\(_{\text{cat}}\) (10–30-fold) on all template-primers assayed. Furthermore, the extent to which k\(_{\text{cat}}\) is diminished, in most instances, is within the range of the total reduction in polymerase activity (5–50-fold), suggesting that events subsequent to template-primer and dNTP binding predominantly contribute to the overall loss in the polymerase activity of P680G and P680Q.

### First dNTP Incorporation on 46/16-mer Template-Primer

Results from the steady state kinetic analyses indicate the effects associated with k\(_{\text{cat}}\) and account almost solely for the
Fig. 2. Determination of the relative DNA binding affinity $K_{\text{rel(DNA)}}$ by UV-mediated cross-linking. 5'-32P-labeled hairpin 37-mer DNA (final concentration of 5 nM) was incubated with the geometrical series increasing concentrations ranging from 5 to 3125 nM of WT (left panel) or mutant KF (right panel), and irradiated at 254 nm as described under “Experimental Procedures.” The E-TP covalent complex was resolved on an SDS-8% polyacrylamide gel and visualized by phosphorimaging. The relative band intensities were quantitated using ImageQuant and plotted against enzyme concentration as shown in the inset. “Relative DNA binding affinity” was calculated from the curve fit to the data using GraphPad PRISM (GraphPad Software Inc). $K_{\text{rel(DNA)}}$ is defined as the concentration of WT or mutant KF that gives 1/2 $B_{\text{max}}$.

| Enzyme | $K_{\text{rel(DNA)}}$ | $K_{\text{rel(mutant)}}/K_{\text{rel(WT)}}$ |
|--------|----------------------|------------------------------------------|
| WT     | 12                   | 1.0                                      |
| P680G  | 19                   | 1.6                                      |
| P680Q  | 5                    | 0.4                                      |
| I679A  | 4                    | 3.0                                      |
| I679L  | 10                   | 0.8                                      |
| N678A  | 35                   | 2.9                                      |
| Q677A  | 37                   | 3.1                                      |
| N675A  | 20                   | 1.7                                      |

TABLE II

Relative DNA binding affinity ($K_{\text{rel(DNA)}}$) for wild type and mutant Klenow enzymes

The relative DNA binding affinity $K_{\text{rel(DNA)}}$ for WT and mutant derivatives of Klenow fragment were determined by plotting the intensity of the covalently cross-linked E-TP species shown in Fig. 2. $K_{\text{rel(DNA)}}$ is defined as the concentration of KF required to give 1/2 $B_{\text{max}}$.

loss of polymerase function by J-helix mutants P680G and P680Q. In order to investigate this step under kinetically simplified conditions, we measured $k_{\text{cat}}$ by providing the first complimentary nucleotide under conditions of excess labeled 46/16 template-primer, as described under “Experimental Procedures.” This effectively removes complications associated with the enzyme translocating along the template-primer, as would be the case when all four nucleotide substrates are present. However, because of the large excess of TP, the dissociation and association with the template-primer are likely to influence the observed rates. Therefore, the measured $k_{\text{cat}}$ values may represent the combined effect of many repeat steps, consistent with the steady state values reported in Table III. Results presented in Fig. 3 show that the WT, N675A, N678A, and I679L add a single nucleotide with approximately equal efficiency, consistent with the kinetic parameters for most of the side chain substituents in this region, with the exception of P680G, which appears to be defective in this process. Quantitation of the relative band intensities for the individual protein yield(s) $k_{\text{cat}}$ values in the range of 1.2 and 0.2 s$^{-1}$ for WT and P680G, respectively. These values are somewhat lower than those presented in Table III for 49/18-mer TP. However, they are consistent with the relative decrease observed for 49/18 (6-fold on 46/16 compared with 8.6-fold on 49/18). Similar experiments were carried out on the labeled 49/17 template-primer, requiring dATP as the first nucleotide to be incorporated in presence of excess WT and mutant KF. The WT KF was found to convert 17-mer to 18-mer product within the first 5 s, whereas, in the case of the P680G(Q) mutants, more than 80% of the labeled primer remained as unextended 17-mer, even after 25 s (data not shown). These results confirm that the defect in the case of P680G(Q) is not restricted to dTTP, but extends to other substrates as well. Since the binding affinity for TP, as judged by UV cross-linking, shows no change for these mutants, we conclude that one or more steps comprising $k_{\text{cat}}$ are rate-limiting for the P680G(Q) mutant proteins.

Processivity of P680G—Processivity is the mean number of nucleotides incorporated in a single enzyme-DNA encounter prior to dissociation. KF is known to be a moderately processive enzyme that adds 10–20 nucleotides per round of DNA synthesis depending on the template-primer. An examination of the products synthesized by P680G with poly(dA)·(dT)$_{18}$, under standard assay conditions, indicated that the products of much shorter lengths were consistently formed in comparison with the wild type (data not shown). This observation suggested that the processive nature of DNA synthesis may be severely compromised by P680G mutation. In order to confirm that the mutant P680G could not synthesize DNA on this template-primer in a processive manner, we performed a time course of incorporation of dTTP on poly(dA)·(dT)$_{18}$, A and B. The results are given in Fig. 4, A and B. In the absence of excess template-primer, the enzyme incorporates multiple substrate dTTP, although at reduced rates. In contrast, in the presence of 100-fold excess of unlabeled template-primer, P680G is able to catalyze addition of only a single nucleotide onto the primer strand. Furthermore, the increase in the +1 product with time was clearly noted, confirming that the enzyme was dissociating from the DNA after the individual nucleotide incorporation and was catalyzing synthesis in the distributive manner.

3’–5’-Exonuclease Activity of WT and Mutant KF on Single-stranded DNA—The initial assessment of nucleolytic hydrolysis by the WT and mutant enzymes was carried out using 5’-labeled single-stranded primer of the 46/16 template-primer (Chart I). In these experiments, both the WT and mutants have exonuclease+ background (see “Experimental Procedures”), and the labeled ssDNA was diluted with 100-fold excess of the same non-radioactive DNA to generate a 1:100 ratio of enzyme:DNA. The reaction was carried out for 1, 2, 5, 10, and 20 min, and the hydrolysis products were resolved on a urea-16% PAGE. The results of this experiment, depicting hydrolysis of a single-stranded 16-mer by the WT enzyme and its mutant derivatives Asn-675, Asn-678, and P680G, are shown in Fig. 5A. A plot of the fraction of 16-mer remaining with time is
The steady state kinetic parameters for the WT and indicated mutant derivatives of Klenow fragment were measured with the indicated template-primer substrates as described under “Experimental Procedures.” 49/21-mer is a synthetic oligomeric template-primers (see Chart I).

| Enzyme      | (dA36)/(dT18) | (dT36)/(dA18) | (dC60)/(dG18) | 49/21-mer | Template-primers used |
|-------------|--------------|--------------|--------------|-----------|----------------------|
|             | $K_m$ $k_{cat}/K_m$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ | $k_{cat}/K_m$ |                      |
| WT          | 7 3 40 x 10^4 | 9 6 70 x 10^4 | 4 0.4 10 x 10^4 | 25 6 20 x 10^4 | (dTTP)               |
| P680G       | 17 0.2 1 x 10^4 | 12 12 3 x 10^4 | 5 0.06 1 x 10^4 | 50 0.2 4 x 10^4 | (dA36)/(dT18)      |
| P680G       | 17 0.2 1 x 10^4 | 12 12 3 x 10^4 | 5 0.06 1 x 10^4 | 50 0.2 4 x 10^4 | (dTTP)               |
| Q677A       | 17 0.2 1 x 10^4 | 12 12 3 x 10^4 | 5 0.06 1 x 10^4 | 50 0.2 4 x 10^4 | (dA36)/(dT18)      |

The template-primer concentration was saturating and dNTP variable as described under “Experimental Procedures.”

b The units of $k_{cat}/K_m$ are $s^{-1}$. ND represents not determined.

catalytic role of J-helix of pol I

Kinetic constants for the polymerase reaction catalyzed by the wild type and mutant Klenow fragment

The relative catalytic ability of mutant KF was assessed by the extent of addition of the first template-directed nucleotide (dTTP) on labeled 46/16-mer template-primer (see Chart I) as described under “Experimental Procedures.”

The first complementary dNTP (TP) was provided to a preincubated KF:TP complex in the presence of a 10-fold excess of enzyme to limit observations to a single turnover (for WT). Products were resolved on a denaturing polyacrylamide gel, visualized by phosphorimaging and the band intensities quantitated using Image Quant (Molecular Dynamics Inc.). The rate constant for dTP incorporation were calculated from the slope of the curves. The results show that P680G and N678A mutants hydrolyze the mismatched nucleotide from the primer termini an order of magnitude faster than the WT enzyme. However, the qualitative examination of the products in the initial period of 5 min (lanes 1–3 indicate 5–25 s reaction time (at 5 s intervals). For P680G, and Q677A lanes 1–5 indicate 20, 40, 60, 120, and 180 s reaction time. The arrow marks the position of unreacted 46/16-mer and lane Ctrl denotes a control reaction carried out with WT but for 20 min to assess the extent of duplex 46/16-mer formation. Quantitation of 16-mer remaining in control lane indicates that more than 90% of 16-mer had been extended to 17-mer product. The lane marked P denotes template-primer without enzyme added.

Fitted to a single exponential, as shown in Fig. 5B. The rate of hydrolusy conversion expressed as $k_{exc}$ determined from these plots, is presented in Table IV. The results shown in Fig. 5 and the rate constants for WT and mutant enzymes indicate that mutation of Pro-680 to Gly, Asn-678 to Ala, and Asn-675 to Ala did not alter the exonuclease activity of KF on ssDNA.

Processivity on 26/17(A)-mer Mismatched Template-Primer—In order to understand the role of the J-helix residues in the exonuclease function, we monitored the cleavage of a double-stranded template-primers containing a single mismatch at the primer termini under the reaction conditions described under “Experimental Procedures.” A 26/17(A)-mer (A represents the mismatched nucleotide) was used for this purpose. The exonuclease activity patterns of the WT and mutant enzymes are shown in Fig. 6A. The percent uncleaved 17-mer substrate as a function of time was plotted (Fig. 6B). A plot of the natural logarithmic values of percent un uncleaved 17-mer with time is shown in Fig. 6B. The data were fit to a single exponential, and the rate constants, $k_{cat}$ (shown in Table IV), were calculated from the slope of the curves. The results show that P680G and N678A mutants hydrolyze the mismatched nucleotide from the primer termini an order of magnitude faster than the WT enzyme. However, the qualitative examination of the products in the initial period of 5 min (lanes 1–3 indicate 5–25 s reaction time (at 5 s intervals). For P680G, and Q677A lanes 1–5 indicate 20, 40, 60, 120, and 180 s reaction time. The arrow marks the position of unreacted 46/16-mer and lane Ctrl denotes a control reaction carried out with WT but for 20 min to assess the extent of duplex 46/16-mer formation. Quantitation of 16-mer remaining in control lane indicates that more than 90% of 16-mer had been extended to 17-mer product. The lane marked P denotes template-primer without enzyme added.

Fitted to a single exponential, as shown in Fig. 5B. The rate of hydrolusy conversion expressed as $k_{exc}$ determined from these plots, is presented in Table IV. The results shown in Fig. 5 and the rate constants for WT and mutant enzymes indicate that mutation of Pro-680 to Gly, Asn-678 to Ala, and Asn-675 to Ala did not alter the exonuclease activity of KF on ssDNA.

3′–5′-Exonuclease on 26/17(A)-mer Mismatched Template-Primer—In order to understand the role of the J-helix residues in the exonuclease function, we monitored the cleavage of a double-stranded template-primers containing a single mismatch at the primer termini under the reaction conditions described under “Experimental Procedures.” A 26/17(A)-mer (A represents the mismatched nucleotide) was used for this purpose. The exonuclease activity patterns of the WT and mutant enzymes are shown in Fig. 6A. The percent uncleaved 17-mer substrate as a function of time was plotted (Fig. 6B). A plot of the natural logarithmic values of percent un uncleaved 17-mer with time is shown in Fig. 6B. The data were fit to a single exponential, and the rate constants, $k_{cat}$ (shown in Table IV), were calculated from the slope of the curves. The results show that P680G and N678A mutants hydrolyze the mismatched nucleotide from the primer termini an order of magnitude faster than the WT enzyme. However, the qualitative examination of the products in the initial period of 5 min (lanes 1–3 indicate 5–25 s reaction time (at 5 s intervals). For P680G, and Q677A lanes 1–5 indicate 20, 40, 60, 120, and 180 s reaction time. The arrow marks the position of unreacted 46/16-mer and lane Ctrl denotes a control reaction carried out with WT but for 20 min to assess the extent of duplex 46/16-mer formation. Quantitation of 16-mer remaining in control lane indicates that more than 90% of 16-mer had been extended to 17-mer product. The lane marked P denotes template-primer without enzyme added.

Processivity of various J-helix mutants was investigated using time course of dTTP incorporation on poly(dA)·dT18 in the presence of excess KF (A) and excess template-primer (B). DNA synthesis on poly(dA)·dT18 was carried out in the presence of excess (100-fold) KF for 40 s at 5-s intervals as described under “Experimental Procedures.” Product formation was monitored by sequencing gel analysis of labeled dT18. The arrow marks the position of labeled primer. E shows the time course of TTP addition onto the 5′-primer-labeled poly(dA)·dT18 in the presence of a 100-fold excess of unlabeled poly(dA)·dT18 over the enzyme to ensure saturation of the enzyme with DNA. The synthesis was monitored at 10-s intervals (data for Ile-679 mutants, which are identical to WT are not shown). The processive (or distributive) mode of DNA synthesis by Pro-680 mutants was inferred from the observation that in presence of excess template-primer, the accumulation of only 1 product is evident implying only a single productive E-TP encounter. In contrast, with excess P680G (A), increase in the higher length products with time is clearly seen. The position of labeled dT18 is marked.

Of Fig. 6A shows that the WT and the mutant enzyme cleave the mismatched substrates to produce different length products. The major product of all the enzymes, with the exception of P680G, is a 17-mer representing correctly paired substrate. P680G, on the other hand, cleaves the single mismatched....
The biochemical data presented here suggest that both DNA binding affinity and $k_{cat}$ did not change significantly upon mutation of Pro-680 to Gly or Gln. The results of steady state kinetic analysis indicated that the reduction in the activity of Pro-680 mutants is proportional to the observed decrease in $k_{cat}$. Therefore, it seems that after the substrate binding step,
the events contributing to $k_{cat}$ may have become rate-limiting in the overall polymerase reaction sequence of Pro-680 mutant enzymes. The $k_{cat}$ under these conditions is mainly controlled by the conformational change and bond formation steps. However, comparison of the binary, open ternary, and closed ternary complexes of KlenTaq polymerase shows that the conformation of J-helix residues and the orientation of side chains of the J-helix residues does not change from binary to open or closed ternary complex formation (7, 8). This indicates that, most likely, the J-helix may not directly participate in the conformational change step of the enzyme-catalyzed polymerase reaction.

Further insight into the functional contribution of the J-helix comes from the observation that P680G/P680Q exhibits a distributive mode of DNA synthesis as judged by the accumulation of only +1 product in a primer extension reaction with excess template-primer (Fig. 4). However, significant reduction in the rates of accumulation of the +1 product or that of total product synthesized in the presence of limited template-primer (Fig. 4B) indicates that a step at or before nucleotide incorporation (chemistry) is also defective. Thus, the effect of mutation of Pro-680 on the polymerase function may be summed up as 2-fold: (a) defect in turnover of bound nucleotide and (b) translocation along the template strand. Consequently, the involvement of the J-helix region in these functions may be suggested.

The two effects described above may have arisen from the disturbance in two distinct physical interactions involving the J-helix. First, the spatial alteration in the position of Gln-677, which is a member of the H-bonding network in the active center, is likely to interfere with the functioning of the H-bonding track. This, in turn, may be scored as a defect in the conformational change or in the chemistry of the bond formation. Second, the fact that Asn-675 and Asn-678 directly interact with the bases of DNA and the backbone structure of J-helix “snugly” fits in the minor groove of the bound template-primer indicates that this region may serve as a “primer grip.” Mutagenesis to the residues of the similar primer grip region of human immunodeficiency virus, type I, reverse transcriptase has been reported to alter severely the processivity of that enzyme (21, 22).

To assess the role of the J-helix region in the exonuclease function, we examined the exonuclease activity patterns of P680G, I679A, N678A, Q677A, and N675A. These mutant enzymes were generated in the exonuclease-positive background. Examination of exonuclease activity with conventional ssDNA substrate did not show any change in the activity of mutant enzymes compared with WT (Fig. 5). However, on a template-primer containing single mismatch at the primer terminus, the rate constants for the exonuclease activity of P680G and N678A were found to be about 10-fold higher than that of WT enzyme. In addition, the major exonucleolytic product for most of the enzymes, with the exception of P680G, appeared to be a double-stranded DNA (±1 nucleotide), representing correctly paired DNA substrate. P680G exhibited a cleavage deep inside the double-stranded region (Fig. 6). These observations suggest different modes of participation of Pro-680 (representing the J-helix) in the enzyme-catalyzed polymerase reaction.

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2 N. Kaushik, K. Singh, S. Tuske, and M. J. Modak, unpublished results.
The first possibility is that being polymerase-deficient, P680G binds the primer terminus in the exonuclease mode. However, this does not seem to be the case, since Q677A mutant KF, despite being polymerase-deficient, has no significant change in its exonuclease activity compared with the wild type enzyme (see Fig. 6, A and B). The second possibility is the increase in rate of shuttling of the mismatched primer terminus of the duplex to the exonuclease active site or an increased affinity for the mispaired primer at the exonuclease site of the mutant enzyme. Millar and colleagues (24) using fluorescence probe containing template-primer have shown that a matched DNA template-primer partitions to the exonuclease site to a negligible degree compared with mispaired DNA. Pre-steady state kinetic analysis may shed more light on this aspect.

The results presented here on the 3′-5′-exonuclease function of the J-helix mutants together with previously reported structural and biochemical results suggest a possible presence of a conformational change step before the chemistry of the phosphodiester bond hydrolysis (28). Our results of the hydrolysis of the 16-mer ssDNA show that the rate constant and the pattern of product formation with WT and mutant enzymes are similar. Considering that ssDNA preferentially binds to exonuclease site, these results imply that the catalytic event of chemistry is not affected nor is it rate-limiting for all the enzyme proteins examined. In contrast, the rate of hydrolysis on mismatched template-primer is increased by ~10-fold for both P680G and N678A mutants (Table IV). This suggests that step(s) other than chemistry may have become rate-limiting in the WT-catalyzed reaction of hydrolysis. This limit appears to be relieved by P680G and N678A mutations.

We propose that the shuttling of the primer terminus between the polymerase and the exonuclease site may be controlled by the J-helix region, and the structural alteration of J-helix (as is the case with P680G) results in a primer shuttling bias toward the exonuclease site. Structural data on KF and homologous polymerases support this proposal. Superposition of the DNA-bound complexes in polymerase and exonuclease sites suggests that a translation of the double-stranded region of the template-primer occurs during positioning of primer from polymerase to the exonuclease site (29). Plausibly, this translation of the double-stranded region of template-primer and a relatively larger conformational change in the thumb, upon binding of DNA in exonuclease mode compared with polymerase mode, may be regulated by the J-helix region. The J-helix of KF has been suggested to function as a “reading head” that monitors the misincorporation of a nucleotide by pol I-type DNA polymerases (2). This suggestion was based on the structural observation of co-crystal structure of DNA-bound KF, in which the primer terminus was positioned in the exonuclease site. Structural data on KF and homologous polymerases show that a matched DNA template-primer partitions to the exonuclease site of the mutant enzyme. Millar and colleagues (24) using fluorescence probe containing template-primer have shown that a matched DNA template-primer partitions to the exonuclease site to a negligible degree compared with mispaired DNA. Pre-steady state kinetic analysis may shed more light on this aspect.
plays a part in positioning the primer terminus of template-primer either in the polymerase or the exonuclease site. The biochemical data, as reported here, appear to be consistent with this suggestion. Regardless of the actual mechanism, our observations strongly implicate J-helix region in the “sensing function” that probably involves the control of shuttling of primer between the exonuclease and polymerase site.

In summary, the present study has clarified the general role of the J-helix region of pol I type of enzymes. In addition to serving as a “primer grip” by aligning peptide backbone with DNA in the polymerase mode, two of its constituents also appear to participate in the polymerase and exonuclease function. Furthermore, the regulation of polymerase and exonuclease function also appears to reside in this region.

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