Phe 771 of *Escherichia coli* DNA Polymerase I (Klenow Fragment) Is the Major Site for the Interaction with the Template Overhang and the Stabilization of the Pre-Polymerase Ternary Complex†

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Received August 21, 2002; Revised Manuscript Received February 12, 2003

ABSTRACT: To identify the sites in the Klenow fragment of *Escherichia coli* DNA polymerase I that interact with the ssDNA overhang of the template strand in the pre-polymerase ternary complex, we carried out UV-mediated photo-cross-linking of the enzyme−DNA−dNTP ternary complex. The template strand contained a nine-nucleotide overhang and was radiolabeled at the 5′-end. Since the enzyme−TP−dNTP ternary complex but not the E−TP binary complex is stable at high ionic strengths, the cross-linking was carried out in the presence of 0.5 M NaCl. The cross-linked E−TP−dNTP complex was purified and subjected to trypsin digestion. The radiolabeled TP cross-linked peptide was further purified by DEAE-Sepharose and C18 column chromatography and subjected to amino acid sequencing. The release of radiolabeled DNA during each sequencing cycle was also monitored. The sequencing results as well as the radioactivity release pattern show that F771, contained in a peptide spanning amino acids 759−775 of pol I, is the unequivocal site of the template cross-linking. A qualitative assessment of the cross-linking efficiency of the template overhang containing a TT sequence at different positions in the ternary complex further suggests that the major cross-linking site within the template overhang is at the second and/or third nucleotide. An examination of the F771A mutant enzyme showed that it was able to form the E−TP binary as well as E−TP−dNTP ternary complex; however, it could not cross-link to the template-primer in the ternary complex. Furthermore, the ternary complex with F771A was qualitatively defective and exhibited some salt sensitivity. These results suggest that F771 participates in the stabilization of the pre-polymerase ternary complex.

Biochemical, genetic, and structural investigations of *Escherichia coli* DNA polymerase I and other related polymerases have contributed significantly to our understanding of the DNA polymerase mechanism and the fidelity and processivity of DNA replication (*1*). Klenow fragment (KF) of *E. coli* DNA polymerase has been used as a model enzyme in understanding the structure−function relationship of nucleic acid polymerases. This 68 kDa polypeptide contains two of the three functions of the entire pol I.[1] The 5′−3′ polymerase activity is located in the C-terminal domain, whereas the 3′−5′ exonuclease activity is on the N-terminal domain of KF (*2*). The crystal structure of KF (*3*) shows that it assumes a half-open right-hand-like structure constructed by palm-, thumb-, and fingers-like subdomains. The active site is located on the palm subdomain in the cleft generated by these three subdomains.

Crystal structures of several DNA polymerases have been reported either in the form of template-primer-bound binary (E−TP) or template-primer- and dNTP-bound ternary (E−TP−dNTP) complexes (*4*−*16*). These crystal structures provide extremely valuable information regarding the stepwise functioning of DNA polymerases. In addition, they also extend the support to the biochemical observations (*17*).

Despite the fact that the crystal structures provide extensive information regarding the protein−DNA contacts that mediate the interactions between the double-stranded region of the template-primer and the polymerase enzyme, the location and the interactions between the single-stranded template overhang and the enzyme and their nature remain unclear. The major reason for the lack of this information is unordered electron density corresponding to the single-stranded region of the template overhang, which is apparent from the higher temperature factor (>20) of this region in the crystal structure (*13*). In addition, the binary or the ternary complex structures have a limited number of nucleotides (only two or three) in the template overhang. Furthermore, the position of the nucleotide upstream of the nascent base pair between the template base and the incoming dNTP is radically bent or "flipped" away from the active site in addition to a significant variation seen in its position in different crystal structures. For example, in the four crystal structures of KlenTaq, a

† Supported in part by a grant from the National Institute of General Medical Sciences (GM 36307).

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[1] Abbreviations: TP, template-primer; E−TP, enzyme−template primer complex; PTH, phenylthiohydantoin; pol I, *E. coli* DNA polymerase I; PAGE, polyacrylamide gel electrophoresis; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; WT, wild type; TEAA, triethylammonium acetate; ss, single-stranded; ds, double-stranded.

10.1021/bi026699y CCC: $25.00
Published on Web 03/14/2003

close relative of pol I, reported in the form of the ternary complex, the position of flipped nucleotide varies from 90° to 180° (*12*, *13*). Thus, neither the region nor the nature of interaction(s) between the ssDNA overhang of the template and pol I is known.

In our investigation of polymerase ternary complex formation of pol I, we confirmed that the template-primer in the ternary complex (E−TP−dNTP) is significantly more tightly bound to the enzyme than it is in the enzyme−TP binary complex (*18*). TP in the ternary complex remains bound to the enzyme at a high ionic strength (0.5 M), whereas a high salt concentration dissociates the E−TP binary complex. Thus, the salt resistance of TP bound to the enzyme in the ternary complex suggests that some hydrophobic interactions exist between the ssDNA overhang of the template and the enzyme. Therefore, the identification of the interacting sites between pol I and TP in the ternary complex may be expected to clarify not only the sites but also the nature of that interaction. We selected KF of *E. coli* DNA polymerase I as a model system for the investigation, since the crystal structure of the ternary complex of this enzyme has yet not been resolved. Furthermore, known properties of the polymerase ternary complex would permit elucidation of regions of this enzyme and TP that are involved in the stabilization of the polymerase ternary complex.

We used a UV-mediated photo-cross-linking approach to generate specific cross-linking adducts of KF and TP under pre-polymerase ternary complex conditions. We have earlier used this procedure to radioactively label the binding site(s) for substrate dNTPs in KF (*19*) and terminal deoxynucleotidyl transferase (*20*, *21*). The labeled enzyme proteins were subjected to tryptic digestion, and amino acid sequence analysis of the purified peptides was used to identify the site of cross-linking. Similarly, the regions involved in the binding of oligomeric primer templates in KF (*22*), MuLV reverse transcriptase (*23*), and HIV reverse transcriptase (*24*) were identified using the cross-linking of the radiolabeled ligand to the desired enzyme followed by amino acid sequencing analysis of the labeled tryptic peptide. The sites of cross-linking identified in the manner described above provided the information concerning the orientation of the base moiety in the binary complexes. The results, i.e., location of cross-linking sites in enzyme protein, are in general agreement with structural data that have since been obtained with crystal structures of some of these proteins (*14*, *25*). For example, a region between amino acids 296 and 305 in HIV-1 RT was identified as the primer-binding region (*24*). The cross-linking of a single-stranded nucleic acid to the C1/C2 HnRNP protein was shown to occur to a specific phenylalanine present in the RNA binding domain of that protein (*26*). Similarly, the cross-linking of dTTP to KF, in the absence of TP, was shown to occur to His 881 (*19*) and that of azido-dATP to Tyr 766 (*27*). These results are generally consistent with the crystal structure data reported for the four individual dNTP-bound complexes of KlenTaq (*25*). The technique of photoaffinity labeling of proteins has been widely used to identify the subunits or regions of proteins involved in the binding of ligands (*28*−*34*). However, the fact that the cross-linking efficiency is extremely low (generally below 1%) makes detailed analysis of the involved site rather difficult. Furthermore, the complexities of isolating the labeled enzyme species free from a large excess of unbound ligand and from the free protein, followed by tryptic peptide mapping of the purified protein and isolation and quantitative analysis by the sequencing of labeled peptide, are technologically challenging and quite labor intensive. It is perhaps for these reasons that many researchers have shied away from this technology and have restricted its use to simple identification of ligand binding subunits or peptides (*34*, *35*). Since our laboratory is one of the few to standardize and successfully utilize this protocol in the past (*36*−*39*), and the specific reaction conditions for radioactively labeling KF with TP have been identified, we felt justified in using this approach to identify the site of the single-strand template overhang in KF. By utilizing the photoaffinity labeling and amino acid sequencing techniques, we show that the cross-linking site between the ssDNA overhang of the template and the enzyme protein is F771. A mutant polymerase containing the F771A substitution was then generated, and its inability to cross-link with the template overhang was confirmed. These observations are further discussed in light of available three-dimensional crystal structures of related polymerases, and a tentative binding track for the single-stranded template overhang, immediately following the template nucleotide, is suggested.

Chart 1

Template-primer used for polymerase activity and kinetic studies

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63/19 mer:
3′- GCAATCGGTGAGGCTTCACGCATATTGCGCGTGCGGCCGCCTGAA….-5′
5′- CGTTAGCCACTCCGAAGTG-3′
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Template-primers used for binary and ternary complex formation assay

33/21 mer dideoxycytidine-terminated primer

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5′-CGTTAGCCACTCCGAAGTGCC-3dd′
3′-GCAATCGGTGAGGCTTCACGGCATATTGCGCGT-5′
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20- mer dideoxycytidine-terminated primer:
5’ –CGTTAGCCACTCCGAAGTGC-3’

30-mer T containing template:
5’-TTTTTTTTGGCACTTCGGAGTGGCTAACG-3’

EXPERIMENTAL PROCEDURES

Materials. DNA-modifying enzymes were purchased from Boehringer Mannheim. *Pfu Turbo* polymerase for PCR amplification was obtained from Stratagene. The dNTPs were from Pharmacia Biotech, while ^{32}P-labeled dNTPs and ATP were from DuPont Perkin-Elmer. TPCK-treated trypsin was purchased from Promega. Biorex 70 cation exchange resin was from Bio-Rad. DEAE-Sepharose was obtained from Pharmacia. All other reagents were of the highest available purity grade and were purchased from Fisher, Boehringer Mannheim, and Bio-Rad. The dideoxycytidine-terminated primer, various templates, primers, and the mutagenic primers for F771A were purchased from MWG Biotech. The sequence of the dideoxycytidine-terminated primer and various 30-mer templates used in this study are as shown in Chart 1. The sequences of other templates used in this study are shown in Figure 4.

Overproduction and Purification of WT and Mutant Enzymes. E. coli DNA polymerase I (Klenow fragment) was

purified from an overproducing exonuclease deficient strain containing the high-level expression plasmid pCJ141, which carries the KF insert with the D424A substitution to abolish the 3′−5′ exonuclease activity (*40*). This plasmid was a kind gift of C. Joyce of Yale University (New Haven, CT). Site-directed mutagenesis was carried out using the plasmid pCJ141 to create F771A. Briefly, mutagenic primers for F771A were used for PCR amplification of pCJ141 using *Pfu Turbo* polymerase, in accordance with the manufacturer's protocol. The amplified product was treated with 10 units of DpnI (37 °C for 2 h) to digest the parental methylated DNA. A 5 μL aliquot of this product was then used to transform the maintenance cell line, *E. coli* CJ406, as described by Sambrook et al. (*41*). The site-directed mutations were confirmed using Sanger's dideoxy termination method for DNA sequencing (*42*). Plasmid DNA from mutant clones was used to transfect *E. coli* CJ376, an expression strain used for this study. Overexpression and purification of WT and the mutein, F771A, were carried out essentially as described previously (*43*, *44*).

Polymerase Activity Assay and Steady State Kinetic Parameter Determination. The polymerase activity of the Klenow fragment and F771A mutein was assayed as described previously using synthetic MS2 63/19-mer TPs and [α-^{32}P]dTTP as the radiolabeled substrate (*37*, *45*). $K_{m.dNTP}$ and k_{cat} were determined using the procedures described previously (*43*).

5′-End Labeling of the 30-mer T-Containing Templates. The 30-mer template containing either a stretch of nine dTMPs or pairwise-positioned dTMP was phosphorylated at the 5′-end using T4 polynucleotide kinase and [γ-^{32}P]ATP. The radiolabeled oligonucleotides were purified by electrophoresis on a 20% acrylamide−7 M urea gel as described by Maxam and Gilbert (*46*) and desalted on a NAP 10 column (Pharmacia).

Pre-Polymerase Ternary Complex Formation Assay. The analysis of the enzyme−TP binary and enzyme−TP−dNTP ternary complexes was performed by gel shift assays. Our method for assessing ternary complex formation is based on the procedure described by Scott and colleagues (*47*), where nondenaturing gel electrophoresis is used to identify the E−DNA−dNTP ternary complex. The TP used in this assay was 30/20-mer DNA that was prepared by annealing of the template and 5-^{32}P-labeled dideoxycytidine-terminated primer (3:1 ratio). The standard binary and ternary complex formation assays in a final volume of 10 μL contained 50 mM Tris-HCl (pH 8), 10% (v/v) glycerol, 10 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 100 pM radioactive TP, and increasing concentrations of enzyme (0.15−1.0 nM). The E−TP binary complexes were formed on ice. The addition of the correct incoming dNTP (100 μM) induced formation of the E−TP−dNTP ternary complex. The stability of the binary and ternary complexes was then analyzed by the degree of persistence of DNA in the original complex, upon challenge with a 500-fold excess of trap (unlabeled identical TP DNA) or with 0.5 M NaCl. We find that under these conditions, DNA from the E−DNA binary complex completely dissociates, while DNA in the ternary complex exhibits significant retention of radiolabeled TP with enzyme protein. All samples were resolved on a native 6% polyacrylamide gel, and the positions of the radioactive bands were visualized by exposure in a PhosphorImager.

Preparative Scale Cross-Linking of the Enzyme to TP in the Ternary Complex and Isolation of the TP-Cross-Linked Protein. A preliminary experiment for determining the optimum ratio of enzyme to radiolabeled TP under the conditions of ternary complexes indicated that a 1:10 ratio of TP to enzyme yielded maximum cross-linking. In these experiments, 5′-^{32}P-labeled 30-mer template strand annealed to 20-mer dideoxycytidine-terminated primer together with 100 μM dCTP and 500 mM NaCl were present. For preparative scale cross-linking, a standard mixture in a final volume of 2 mL contained 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10% glycerol, 15 nmol of 5′- ^{32}P-labeled 30/20-mer dideoxynucleotide-terminated TP, and 100 μM dCTP. The ternary complex was formed by incubating the reaction mixture containing the E−TP complex on ice for 10 min followed by the addition of dCTP. The complex was incubated for an additional 10 min on ice, and NaCl was added to a final concentration of 0.5 M, to dissociate the E−TP binary complex. The reaction mixture was exposed to UV (254 nm) irradiation in a Spectrolinker (Spectronic Corp.) at 3800 μJ/cm^2 for 3 min. An aliquot of the irradiated samples was mixed with a 2× SDS dye solution, heated at 100 °C for 10 min, and resolved by 8% SDS−PAGE. Radioactive bands were visualized by scanning the gel in a PhosphorImager (Molecular Dynamics) and quantitated using ImageQuant software (Molecular Dynamics). After the final NaCl concentration had been adjusted to 0.1 M, the remainder of the preparative scale reaction mix was loaded on a DEAE-Sepharose column equilibrated with 0.1 M ammonium acetate. Cross-linked or un-cross-linked enzyme and TP were bound to the matrix, as neither radioactivity nor protein was detected in the flow-through or 0.1 M wash. The un-cross-linked KF was eluted with 0.3 M ammonium acetate, after which the column was washed with 0.5 M ammonium acetate to remove the traces of free KF. The cross-linked protein was eluted at 0.8 M ammonium acetate, as judged by the radioactivity associated with 0.8 M fractions and subsequent SDS−PAGE analysis. Free DNA bound to the column was eluted with 1 M ammonium acetate.

Trypsin Digestion of the TP-Cross-Linked Protein and Purification of the Cross-Linked Peptide. The radioactivity associated with template DNA allowed quantification of cross-linked products. The 0.8 M ammonium acetate fractions containing cross-linked protein were pooled, concentrated by polyethylene glycol dehydration, and dialyzed against a solution of 1 M urea and 50 mM ammonium bicarbonate (pH 8.5). Trypsin digestion of the dialyzed complex was effected by addition of trypsin, at a protein:trypsin ratio of 20:1. Following trypsin digestion, the TP-cross-linked peptide was purified by DEAE-Sepharose chromatography as described above. The radiolabeled DNA-cross-linked peptide, which eluted in 0.8 M ammonium acetate, was desalted by C18 column chromatography. The matrix-bound peptide was eluted with 50% methanol and 5 mM TEAA (pH 8). An aliquot of the cross-linked peptide was electrophoresed on a 20% polyacrylamide−7 M urea gel and analyzed for its purity and associated free DNA, if any. The purified cross-linked peptide sample was freed from TEAA by repeated washing and lyophilization. Amino acid sequencing of the peptide sample was performed by the William M. Keck Foundation Biotechnology Resource Laboratory at Yale University. The sequencing was carried out with an Applied

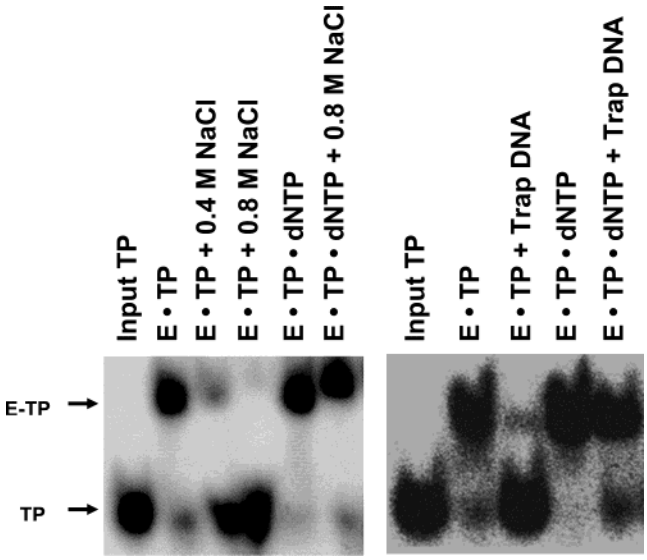

FIGURE 1: Analysis of pre-polymerase E−TP binary and E−TP−dNTP ternary complexes via a gel shift assay in which 5′-^{32}P-labeled TP (30/20-mer ddC) and KF were incubated on ice in a reaction volume of 10 μL. The concentration of TP was 100 pM, while that of KF was 1 nM. For ternary complex formation, dCTP was added at a concentration of 100 μM. The effect of high ionic strengths (0.4 and 0.8 M NaCl) and trap DNA (50 nM nonradioactive 30/20-mer ddC) on the stability of radiolabeled TP was assessed by resolving the complexes on a 6% polyacrylamide gel as described in Experimental Procedures. Note the stability of the E−TP−dNTP complex at high ionic strengths as well as when challenged with trap DNA.

Biosystems model 477 peptide sequencer, equipped with online HPLC. A duplicate sample was manually processed so that products of the individual amino acid cycles were collected and assessed for radioactivity.

RESULTS

E−TP Binary and E−TP−dNTP Ternary Complexes of KF. The binding of TP followed by dNTP, which is complementary to the first template nucleotide, is a prerequisite for the polymerization reaction by DNA polymerases. The two binding events are termed binary and ternary complex formation, respectively. Since the dNTP in the ternary complex is rapidly turned over, use of a dideoxynucleotide-terminated primer in these complexes permits formation of a stable pre-polymerase ternary complex. The binding of TP in two complexes, however, represents two different conformations of the enzyme protein, in that TP in the ternary complex is dissociated extremely slowly when challenged with a 500-fold excess of free TP, whereas TP in the binary complex readily exchanges with the challenge TP (*18*). Figure 1 depicts typical KF−TP binary and KF−TP−dNTP ternary complexes and the effect of challenge DNA on the stability of TP in the original complexes. In addition, TP in the binary and ternary complexes also displays differential sensitivity to 0.5 M NaCl, in that TP in the ternary pre-polymerase complex is not dissociated, while the one in the binary complex is completely dissociated from the enzyme (Figure 1). These observations provided an opportunity to unambiguously determine the binding site(s) in KF for TP in the ternary complex.

Cross-Linking of TP to KF in the Binary and Ternary Complexes. Since the salt resistant binding of TP in the ternary complex requires the presence of complementary dNTP, it was reasonable to assume that the single-stranded region of the template was involved in the stabilization of the ternary complex. We adapted the photoaffinity labeling approach to generate covalent adducts between nucleotide(s) of the template and their contact points (amino acids) in the enzyme protein. The 3′-dideoxycytidine-terminated 20-mer primer annealed to a 5′-^{32}P-labeled 30-mer template (see Materials in Experimental Procedures) provides G as the templating base, and therefore, addition of 100 μM dCTP promotes ternary complex formation. A typical labeling pattern obtained with 30/20-mer ddC TP under binary or ternary complex conditions and in the presence or absence of 0.5 M salt is shown in Figure 2. It is clear from Figure 2 that radiolabeled TP in both binary and ternary complexes cross-links to a similar extent. However, in the presence of 0.5 M NaCl, no cross-linking of TP to the enzyme in the binary complex is apparent. In contrast, cross-linking of TP to the enzyme in the ternary complex remains nearly unaffected. We, therefore, conclude that the UV-mediated cross-linking of TP to the enzyme in the presence of 0.5 M NaCl represents specific binding of TP in the ternary complex conformation.

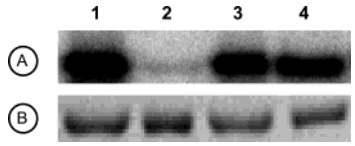

FIGURE 2: UV-mediated affinity labeling of KF with a template under binary and ternary complex formation conditions and the effect of 0.5 M NaCl. In the standard irradiation mixture, 3 pmol of 5′-^{32}P-labeled 30-mer template annealed to 20-mer primer containing 3′-ddC was mixed with 30 pmol of KF to generate the E−TP binary complex (lane 1). The addition of 100 μM dCTP converts the binary complex into the ternary complex (lane 3). The two complexes were then exposed to 0.5 M NaCl (lanes 2 and 4, respectively) and irradiated as described in Experimental Procedures. The cross-linked samples were resolved by 8% SDS−PAGE. (A) PhosphorImage and (B) Coomassie blue staining pattern of the same gel. Note the persistence of the E−TP−dNTP ternary complex in the presence of 0.5 M NaCl (lane 4).

Preparative Scale Cross-Linking of the E−TP Complex in the Ternary Complex, Purification of the Cross-Linked Protein, and Tryptic Digestion. The pilot experiments with varying ratios of 30/20-mer ddC to KF (ranging from 1:1 to 1:20) indicated that optimal cross-linking of the 30-mer template to the enzyme in the presence of 100 μM dCTP (a ternary complex formation condition) and 0.5 M NaCl occurs at a ratio of 1:10. The efficiency of the UV-mediated cross-linking reaction with unmodified TP is rather low since no photoactivatable analogue or modified nucleotide was used. The use of the latter is likely to cause labeling of sites slightly away from the absolute nucleotide interacting sites. We therefore preferred and relied on the ability of the natural base moiety of DNA to induce cross-linking. Indeed, the choice of a T-enriched template overhang was based on the fact that T nucleotides are the best cross-linkers among the four nucleotides (*48*). The yield of the cross-linked ternary complex made with 150 nmol of KF and 15 nmol of TP was approximately 3%. The UV-irradiated reaction mixture after 1:5 dilution was processed on a DEAE-Sepharose column. Free enzyme was eluted from the matrix at 0.3 M salt, while E−TP−dNTP complexes were enriched in a

Table 1: Identification of Amino Acids and Radiochemical Sequencing of the Peptide Cross-Linked to the Template Overhang[a]

sequencing cycle	deduced amino acid	residue position in pol I	yield of PTH derivative (pmol)	associated radioactivity (cpm)
1	Ala	759	32.07	1722
2	Ile	760	32.19	879
3	Asn	761	14.00	914
4	Phe	762	22.59	720
5	Gly	763	6.8	491
6	Leu	764	16.91	396
7	Ile	765	26.31	309
8	Tyr	766	16.01	293
9	Gly	767	4.83	232
10	Met	768	12.36	215
11	Ser	769	2.57	195
12	Ala	770	11.45	155
13	Phe	771	1.79	409
14	Gly	772	2.51	381
15	Leu	773	6.06	313
16	Ala	774	6.13	126
17	Arg	775	1.04	205

[a] The tryptic peptide covalently linked with the template overhang was selectively purified by DEAE-Sepharose chromatography as described in Experimental Procedures. The purified peptide was subjected independently to radiochemical as well as gas phase sequencing. The radioactivity released and the yield of PTH drivatives in each cycle were recorded. The table depicts the amino acid identified in each cycle, its position in the primary sequence of pol I, the picomole yield of its PTH derivative, and the associated radioactivity.

fraction eluting with 0.8 M salt. Free TP was eluted at 1 M salt. The radioactivity monitoring as well as SDS−polyacrylamide gel profile of various fractions permitted the identification of E−TP−dNTP species. The 0.8 M fractions were concentrated and subjected to trypsin digestion, and tryptic peptide(s) containing cross-linked DNA was further purified on a DEAE-Sepharose matrix. The radiolabeled cross-linked peptide obtained in this manner, after desalting on a C18 matrix, was processed for amino acid sequencing as well as release of radioactivity in individual cycles. The final yield of the cross-linked peptide was ~300 pmol. A small aliquot of this peptide fraction was also resolved on a 20% polyacrylamide−7 M urea gel. A single radioactive band migrating slower than 30-mer template DNA was consistently observed (data not shown). Thus, there appeared to be a single peptide cross-linked to the template overhang. The specificity of the template-cross-linked peptide is also apparent from the observation that no labeling occurred when dNTP was omitted from the ternary complex reaction mix that contained 0.5 M NaCl or when the complete reaction mix was not exposed to UV irradiation.

Amino Acid Sequencing and Release of Radioactivity from the Cross-Linked Peptide. Amino acid sequence analysis carried out with approximately 150 pmol of peptide unambiguously identified a single tryptic peptide of 17 amino acids, from Ala 759 to Arg 775 in the primary amino acid sequence of pol I. There was no indication of any secondary sequence, suggesting that the 17-amino acid peptide is the only peptide cross-linked with DNA. The amino acid identified in each cycle and the yield of the PTH amino acid derived in that cycle are shown in Table 1. The yield of glycine in each of its corresponding cycles is low compared to the yields of the neighboring amino acids. However, because three different glycine sites showed a low yield, none of these are likely to represent the cross-linking sites.

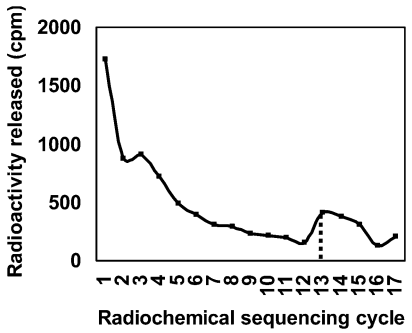

FIGURE 3: Radiochemical sequencing of the peptide cross-linked to the template overhang. The release of radioactivity during each individual sequencing cycle (see the footnotes of Table 1) is graphically depicted. The dotted line indicates an increase in the amount of radioactivity released in the 13th cycle (Phe 771 of the 17-mer peptide) over the previous cycle.

Similarly, the yield of Ser at position 769 (cycle 11) is low; however, the yield of PTH-Ser is typically only 10−15% of that of most other PTH derivatives (*49*). Furthermore, no radioactivity release above the background level (i.e., more counts per minute released over the previous cycle) was observed (see below) in all cycles corresponding to Gly positions in the peptide (5, 9, and 14) as well as in cycle 11 (corresponding to Ser 769 in the tryptic peptide). The 13th sequencing cycle (position 13 in the peptide) was nearly blank, and therefore, no amino acid assignment could be made. A very small amount of Phe was detected in this position, as listed in the known sequence, but this quantity was much smaller than the amounts of the surrounding amino acids (~16% of Ala in position 12). Taken together with the radiochemical sequencing data, position 13 in the tryptic peptide, identified as Phe 771 in KF, is assigned as the unambiguous site for the cross-linking with the template overhang.

Radiochemical Sequencing of the Cross-Linked Peptide. Since we used a 5′-^{32}P-labeled 30-mer oligonucleotide as a template in the UV-mediated affinity labeling of KF, the identification of the peptide was accomplished simply by following its radioactivity. Therefore, an increase in the level of radioactivity released during peptide sequencing may be expected to occur upon the release of the residue that has cross-linked to the 5′-^{32}P-labeled 30-mer template. Experimentally, approximately 8000 cpm containing the cross-linked peptide sample (~150 pmol) was processed on an automated sequencer, and total reaction products of each cycle were manually collected. The majority of the radiolabel was detected in the first three cycles, which most likely represents the breakdown of DNA (Figure 3). However, release of radioactivity from cycle 3 till cycle 12 continued at a decreasing level. Interestingly, an increase of ~2.6-fold in cycle 13 (409 cpm), which is the position of Phe 771 in the peptide, was noted. In subsequent cycles, a steady decrease in the level of radioactivity from this level was observed (Figure 3). Determination of the amount of radioactivity contained in the individual cycle of sequencing has not been successfully reported prior to this report. Because of the rather harsh chemical environment that cross-linked DNA encounters during the successive sequencing cycles, we had expected that the majority of cross-linked DNA may be degraded or lost in the first few cycles of sequencing. However, even if a small fraction of DNA

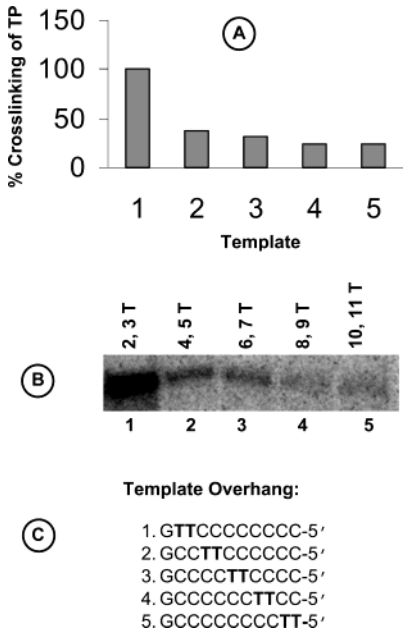

FIGURE 4: Effect of the positions of thymidine in the template overhang on cross-linking with KF. The 31-mer templates containing the desired sequence of the overhang marked 1−5 in panel C were labeled with ^{32}P at their 5′-ends and annealed to a 20-mer ddC primer (as described in Experimental Procedures). The individual template overhang contained a pair of thymidines at the indicated positions. The degree of interaction(s) of each individual template overhang with KF in the ternary complex form was then assessed by UV-mediated photoaffinity lebeling. Panel B shows the cross-linked complexes resolved by 8% SDS−PAGE. The numbers on top of panel B represent the respective position of thymidine (T) in the single-stranded region of TP. The percentage of cross-linking with TP containing thymidine at positions 2 and 3 was taken to be 100, and cross-linking of other TPs was normalized accordingly. The effect of the position of thymidine in the template overhang on cross-linking (and thus the inferred interaction with the residues in KF) is depicted graphically in panel A.

survives the extremes of acid environment during sequencing, it may provide a qualitative indication of its association with a specific amino acid. It may be pointed out here that when 30-mer DNA containing 100 000 cpm was irradiated and mixed with the tryptic digest of KF and processed in a manner similar to that of the cross-linked peptide, all the radioactivity was released in the first three cycles. On the basis of these results, we consider that the radioactivity released in cycle 13 provides an indication of association of the amino acid contained in that cycle with the 30-mer template.

Identification of the Position of the Nucleotide in the Template Overhang Onteracting with Phe 771 of KF. The template overhang used to identify Phe 771 as the cross-linking site contained an overhang of nine thymidines in the 5′-region. To further address which of these thymidines was interacting with Phe 771, various 31-mer templates containing a pair of thymidines at the desired location in the single-stranded region were used. The remainder of the single-stranded overhang consisted of deoxycytidine residues. The various templates containing two successive thymidines at positions 2 and 3, 4 and 5, 6 and 7, 8 and 9, or 10 and 11 (see Figure 4) were synthesized and labeled with ^{32}P at their 5′-end. After annealing with the 20-mer dideoxycytidine-terminated primer had been carried out (1:3 ratio), individual TP was used for cross-linking experiments with wild-type KF under E−TP−dNTP ternary complex conditions. As shown in Figure 4, maximum cross-linking was observed when the thymidine was positioned as the second and third nucleotide in the template overhang. Significantly less cross-linking was observed when the thymidines were positioned at the fourth and fifth nucleotide in the template overhang, while shifting the position of thymidine beyond the fourth and fifth nucleotide in the template overhang resulted in low yields. Thus, the cross-linking data suggest that the preferred site of cross-linking in the template overhang resides in the second and/or third nucleotide. Since Phe 771 is the only amino acid identified as a site of interaction between the template DNA overhang and protein, we concluded that the interaction of Phe 771 occurs with the second and/or third nucleotide in the template overhang.

Table 2: Comparison of Kinetic Parameters of Wild-Type Klenow Fragment and F771A[a]

enzyme	DNA polymerase activity (%)	$K_{D.DNA}$ (nM)[b]	$K_{D.DNA}$ in the ternary complex (nM)[c]	$K_{m.dTTP}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
WT	100	0.55	0.19	3.6	8.5	2.4×10^6
F771A	70	0.98	0.31	3	4.9	1.6×10^6

[a] Wild-type KF and its mutant derivative F771A were analyzed for their activity and steady state kinetic properties using 63/19-mer heteropolymeric DNA. The activity of wild-type KF was taken to be 100%, which was 2.3×10^4 units/mg of protein. The activity of F771A was determined to be 1.8×10^4 units/mg of protein. One unit of enzyme activity is defined as the amount of enzyme activity necessary to incorporate 10 nmol of dNMP into an acid insoluble form in 30 min at 37 °C. [b] $K_{D.DNA}$ was determined using the 33-mer template DNA annealed to the 20-mer ddC terminated primer. [c] $K_{D.DNA}$ for the ternary complex was determined in the presence of both 33/20-mer ddC template primer and 100 μM dGTP (the incoming nucleotide). For both the binary and ternary complexes, the extent of DNA in the complex was determined from the amount of free DNA left in the individual binding reaction mixture (*52*). The complexes formed with F771A show significant dissociation of TP during the electrophoresis, which appears as a streak on the gel.

Analysis of the Interactions of F771A KF with the Template Overhang in the Binary and Ternary Pre-Polymerase Complexes. The results described above identified Phe 771 of pol I as the cross-linking site, which interacts with the second and/or third nucleotide of the template overhang. To further clarify the role of Phe 771 in this process, we generated a F771A mutant KF, and assessed its ability to form binary and ternary complexes, as well as its ability to cross-link to template DNA under two conditions. Preliminary characterization of the F771A mutant protein showed an ~30% loss of catalytic activity, with no change in $K_{m.dNTP}$ and a slight change in $K_{D.DNA}$ compared to those of the wild-type enzyme (Table 2). The $K_{D.DNA}$ was reduced for both WT and F771A in the presence of the correct dNTP (ternary complex formation condition), indicating that F771A can form the E−TP−dNTP ternary complex. Furthermore, F771A exhibited cross-linking to 30/20-mer ddC TP at approximately the same level as WT (Figure 5A). Thus, E−TP binary complex formation did not seem to require the participation of F771. As expected, binary complex formation was completely abolished in the presence of 0.5 M NaCl (Figure 5B). Most interestingly, very little cross-linking of TP to F771A was apparent when TP was bound in the ternary complex mode (Figure 5B), and the presence of 0.5 M NaCl did not change this property. Direct examination of F771A in forming a pre-polymerase ternary

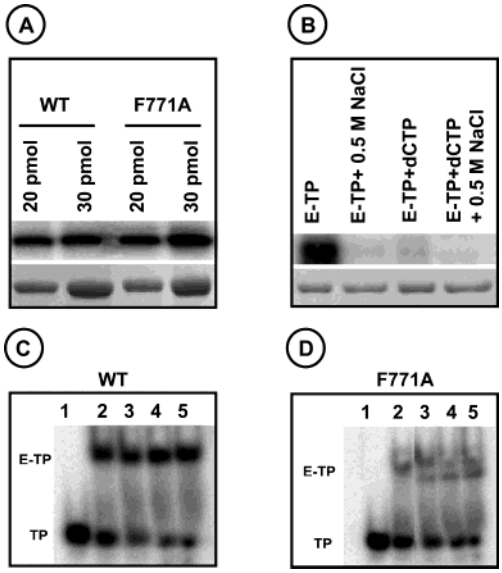

FIGURE 5: TP binding properties of WT and the F771A mutant. Panel A depicts the UV-mediated cross-linking efficiency for cross-linking of the wild-type KF and mutant F771A to TP assessed using 20 and 30 pmol of two enzyme species using a 1:10 TP:E ratio as described in Experimental Procedures. The cross-linked complexes were resolved by 8% SDS−PAGE. No significant difference in the cross-linking ability of the F771A and WT enzyme was noted. The lower half panel shows the Coomassie blue staining pattern representing the amount of protein used in each cross-linking reaction. Panel B shows the UV-mediated affinity labeling of the mutant F771A with TP under binary and ternary complex formation conditions, as well as the effect of 0.5 M NaCl on cross-linking. The conditions for the formation of binary and ternary complexes were similar to those described in the legend of Figure 1. The cross-linked complexes were resolved by 8% SDS−PAGE. Note that no cross-linking is observed in the presence or absence of 0.5 M NaCl when TP is bound in the ternary complex. The Coomassie blue staining pattern of the enzyme used in the cross-linking is shown in the lower half of the panel. Panels C and D show the comparative ability of binary and ternary complex formation by the wild-type and F771A mutant enzymes. In this experiment, E−TP binary complexes were formed as described in the legend of Figure 1 using 0.5 ng of WT enzyme (panel C, lane 2) or 2 ng of F771A (panel D, lane 2) and ~100 pM 5′-^{32}P-labeled 30T/20-mer ddC TP. Lane 3 in panels C and D shows the formation of the ternary complex by addition of 100 μM dATP to the binary complex, while lanes 4 and 5 show the effect of 0.25 and 0.5 M NaCl, respectively, on the ternary complexes. All the complexes were resolved on the nondenaturing 6% polyacrylamide gel, and the quantitation per position of radiolabeled TP was detected by PhosphorImaging (Molecular Dynamics Inc.). Note that F771A does not form stable binary or ternary complexes similar to those seen with WT. However, a slight decrease in the stability of the ternary complexes of F771A is detected in the presence of high ionic strengths (see the free TP band).

complex shows that it can indeed form a ternary complex, although the quality of the ternary complex consistently differed from that of the wild-type enzyme (Figure 5C,D). Generally, both the binary and ternary complexes of F771A appeared to be somewhat unstable since no distinct band of the complex species was observed on the native gels. Furthermore, significant dissociation of preformed complexes appeared to occur during the electrophoresis of the samples. It was therefore necessary to quantitate binding based on the free TP, rather than the TP bound in the complex. The ternary complex of F771A also exhibited slightly increased sensitivity to salt as shown in Figure 5D. The TP bound to F771A in the ternary complex is sensitive to the addition of 0.5 M NaCl as ~45% of the bound TP dissociates from the complex upon salt challenge, whereas only ~20% TP dissociates from WT KF under similar conditions.

DISCUSSION

In a classical DNA polymerase reaction, the enzyme binds to TP, forming a binary complex. This complex then serves as a receptor for the binding of one of the four dNTPs, dictated by the template nucleotide, in forming a pre-polymerase ternary complex. The change from the binary to the ternary complex is accompanied by the rearrangement of a number of residues involved in the initial binding (*12*, *13*). The most important observation from the structural viewpoint is the motion of the O helix region in the ternary complex observed in many pol I-type polymerase structures (*11*). In fact, TP bound in the binary complex appears to become locked in the polymerase cleft upon the binding of the appropriate dNTP. Thus, TP bound in a ternary complex may represent an active intermediate where some of the binding contacts may be different from those of the same TP bound to the enzyme in a binary complex. Since a large body of structural information is available on the pol I family of enzymes and their binary or ternary complexes (*3*, *8*, *11*−*13*), we chose KF of *E. coli* DNA polymerase I as a model enzyme to investigate the binding of TP in its ternary complex form. As shown in Figure 1, KF indeed forms a stable ternary complex consisting of TP, dNTP, and enzyme protein. TP in this complex is tightly bound so that neither competing trap DNA (the same TP) nor the addition of 0.5 M NaCl dissociates the originally bound TP. In contrast, both of these treatments readily displace originally bound TP in the binary complex. One key requirement for demonstrating ternary complex formation is the use of a dideoxy-terminated primer, which permits the binding of dNTP complementary to template nucleotide, but prevents its incorporation or turnover. Thus, our 20-mer primer is a ddCMP-terminated species annealed to a 30-mer, with dCTP serving as a first template nucleotide. Ternary complex formation required addition of dCTP to the reaction mixture. Because of the salt resistant nature of the ternary complex, we surmised that the binding of the single-stranded DNA overhang may be responsible for this property. Furthermore, the double-stranded DNA binding track has been well-documented in many DNA polymeras−-TP cocrystal structures. The binding force in these structures appears to be strictly ionic in nature (*4*−*16*).

The initial assessment of UV-mediated cross-linking of an individually radiolabeled template or primer under the binary or ternary complex conditions showed that the labeled template strand cross-linked at least 3 times more efficiently than the primer strand (data not shown). Both template and primer strand cross-linking require the presence of a full-fledged TP since no significant cross-linking of either strand alone in the presence or absence of dNTP is noted. The effect of a high ionic strength (0.5 M NaCl) on the cross-linking of E to TP in the binary or ternary complex is depicted in Figure 2. Like the stability of the ternary complex upon addition of 0.5 M NaCl, the cross-linking reaction also exhibited salt resistance. No cross-linking of E to TP can, however, occur in the presence of 0.5 M NaCl, suggesting complete dissociation of the binary complex. Thus, selective labeling of the enzyme protein (KF) with the template strand

seemed to be feasible. We had previously used a self-annealing 37-mer as an affinity labeling reagent and had found that the sites of cross-linking of DNA to KF are contained in the same tryptic peptide, spanning residues 759−775. The assignment of actual amino acid residues involved in cross-linking, however, lacked confidence due to a number of analytical and technical problems (*22*, *49*). The only confirmed site of cross-linking identified in that analysis was Tyr 766. On the basis of the rather questionable data, which indicated Ser 769 and Phe 771 as possible sites of cross-linking, we had suggested that tyrosine may be close to the primer terminal nucleotide, whereas Ser 769 or Phe 771 may interact with the template base. Prior to that report, Catalino et al. (*50*), using azido-derivatized primer terminal nucleotide, had shown that the primer terminus cross-linking site in pol I also resided at Tyr 766. In the study presented here, in the ternary complex, no cross-linking of the template strand to Tyr 766 was detected but its cross-linking to F771 was conclusively shown to occur (see below). It is therefore tempting to reiterate our earlier suggestion that primer stabilization may occur at Tyr 766 while template stabilization may be provided by F771.

With the establishment of conditions for selective labeling of the template strand in the ternary complex of KF, we felt confident that identification of the site(s) of template cross-linking in the enzyme protein can be accomplished. To enhance the yield of photolabeled adducts, we introduced nine T residues in the single-strand overhang of the template, which corresponded to template nucleotides 2−10. We had previously reported that T nucleotides are the best cross-linkers among the four nucleotides (*48*). As judged by the input TP, ∼3% of the input DNA is consistently cross-linked to the enzyme protein. As described in detail in Experimental Procedures, the cross-linked protein was isolated from free enzyme and free TP and was subjected to tryptic digestion. The tryptic peptide cross-linked to the template strand upon sequencing clearly showed that a single peptide spanning residues 759−775 contained the cross-linking site, which was further narrowed to F771, a residue occurring at the 13th cycle in that peptide (Table 1). The identification of F771 in the 13th cycle is accomplished by observing that it is a missing residue in the analysis of the labeled peptide. As noted previously (*22*, *49*), yields of PTH derivatives of amino acids in each successive cycle have to be carefully evaluated, for yields of certain amino acids (e.g., glycine and serine) are consistently low. Most importantly, we are reporting the results of our radiochemical sequencing analysis of the same sample monitoring the release of radioactivity (from the labeled product) during each cycle. Because of the extremely acidic conditions employed in the amino acid sequencing protocol, it was expected that the majority of the radiolabeled DNA will suffer chemical breakdown and all the radioactivity will be in the first few cycles. While this expectation was not unreasonable, a small peak of radioactivity at cycle 13 (corresponding to position F771) was indeed observed (Figure 3). We suggest that this information may be qualitative but confirmatory in nature. To assess the site on template DNA that interacts with Phe 771, we used positioning of a pair of T residues in the ssDNA overhang of the template and compared their cross-linking efficiencies. Of the five pairs of T nucleotides located at template nucleotide positions 2 and 3, 4 and 5, 6 and 7, 8 and 9, and 10 and 11, the optimal cross-linking occurs with a T at the second and third positions. These results strongly suggest that Phe 771 interacts with the seconnd and/or third template nucleotide in the ternary complex.

The participation of F771 in the binding and/or stabilization of the template overhang in the "ternary complex" is also supported by the cross-linking studies performed with the F771A mutant enzyme. This mutant (F771A) failed to cross-link to TP in the ternary complex with or without addition of high salt (Figure 5B). However, it exhibited a nearly unchanged level of cross-linking with TP in the "binary complex" compared to that of WT (Figure 5A). F771A also exhibited rather interesting properties with regard to the formation of the binary and ternary complexes. The mutant protein retained ∼70% of the catalytic activity and only slightly altered the binding affinity for TP (as judged by an only ∼2-fold increase in $K_{D.DNA}$, Table 2). Furthermore, F771A appeared to form a ternary complex as inferred from the fact that the $K_{D.DNA}$ in the presence of substrate dNTP did exhibit tighter binding of DNA, a qualitative indication of ternary complex formation (Table 2). However, the quality of the binary as well as the ternary complex formed by F771A was consistently different from that of the wild-type enzyme, as noted during the separation of the complexes on native polyacrylamide gels. While the WT enzyme was able to form a stable ternary complex band, the mutant complexes were found to display a diffused pattern, implying a somewhat unstable nature of these complexes (Figure 5C,D). Moreover, the ternary complex of F771A also exhibited slightly increased sensitivity to the addition of salt, as judged by the fact that nearly 45% DNA from the complex was released from the ternary complex in the presence of 0.5 M salt, whereas the ternary complex formed by the wild-type enzyme released only ∼20% (Figure 5C,D). These results suggest that F771 participates in the formation of the stable ternary complex. However, F771 does not appear to be the only site for stability, since ternary complex formation, albeit of inferior quality, was possible with F771A, implying an additional contact or binding site(s) for the single-stranded overhang.

The stability afforded by the interaction of the second or third template nucleotide with F771 in the ternary complex further suggests that the single-stranded region of the template overhang may be specifically directed away from the preceding dsDNA region, permitting an interaction of the remainder of the single-stranded template with some other region in the enzyme. The binding of the single-strand overhang also appears to follow different paths in different polymerases. For example, in pol I-type enzymes, the overhang seems to stabilize by bending sideways toward the Q helix, while in HIV-1 RT, it passes through the fingers subdomain (*14*). However, the position of the template overhang seen in the crystal structures of several DNA polymerases is not ordered (*11*−*13*). Since the crystal structures of these polymerases have only a limited number of nucleotides in the template overhang, a definite binding track for the single-stranded overhang is not clear. A tentative region in pol I that may be involved in the binding of the single-stranded overhang can be inferred from the analysis of the crystal structures of these polymerases and our present result that the second and third template nucleotide binds with F771. The flipped nucleotides in four ternary complex

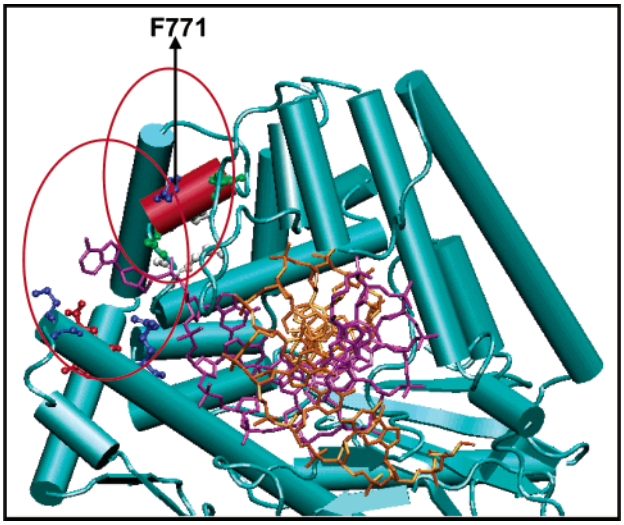

FIGURE 6: Proposed model for the binding of the template overhang in pol I. This figure shows the relative position of F771 and the flipped template nucleotide in the ternary complex of KlenTaq (*12*). This figure has been generated from the ternary complex crystal structure of KlenTaq (PDB entry 3KTQ). The enzyme has been rendered according to the secondary structure (i.e., the α-helices are shown as cylinders and *β*-strands as block arrows) in cyan. Some helices in the polymerase structure are labeled for ready reference. The template (purple) and primer (orange) are shown in stick representations. The two circled regions correspond to the two cavities that are proposed to be involved in the binding of the template overhang (see the text). The lower cavity (1) contains positively charged residues on one surface which most likely binds the phosphate backbone of ssDNA. The other surface of this region mainly contains hydrophobic residues, including F771, and is the major area where base moieties of the template overhang interact. Cavity 2 contains a number of hydrophobic residues, which probably stabilize the helix−turn−helix regions involving O-, O1-, and O2-helices. The residues of the two cavities are shown in ball-and-stick mode and are color-coded. White, blue, red, and green correspond to hydrophobic, basic, acidic, and polar residues, respectively. The residues shown in this figure correspond to those of Taq polymerase I. Thus, H676 occupies the position of F771 of *E. coli* DNA polymerase I. The dotted line shows a possible path for the binding of the phosphate backbone of the template overhang.

crystal structures of KlenTaq appear to be sandwiched in a cavity (hereafter called cavity 1, Figure 6) between the loop (and part of the Q-helix) that connects *β*11 and the Q-helix, and O1-helix, albeit the orientation of the flipped nucleotide in individual ternary complexes is significantly different. There are several conserved residues whose side chains are exposed to this cavity. These conserved residues are M768, S769, F771 (H676 in KlenTaq), L773, R775, R841, and R836. The interesting part of the architecture of this cavity is that both basic and acidic residues line up on its one side (polar side). These residues are R841, R835, R836, and E840. Although R835 of *E. coli* pol I is not conserved in the pol I family, the total number of charged residues on this side of cavity is conserved. For example, in KlenTaq these are R746, E845, R742, and K738. The presence of both positive and negative residues appears to have some functional significance. The positively charged residues may be postulated to bind the phosphate backbone of the single-stranded template overhang. The presence of the negative charge (E840) in the cluster of three positively charged residues (R841, R835, and R836) can be postulated to play a role in the binding and translocation of KF on the single-stranded region. For example, the interaction between the phosphate backbone of the template nucleotide and R841 can be neutralized by formation of an ion pair between R841 and E840. The other site (nonpolar side) of cavity 1 contains a combination of hydrophobic and polar residues capable of forming H-bonds. These residues are M768, S769, F771, L773, and R775 (substituted with Q/N in other pol I enzymes). The residue, which we have identified as the cross-linking site (F771) with the template overhang, is part of this side of the cavity. These structural data suggest that the phosphate backbone and base moieties of the template overhang are stabilized by polar and nonpolar sides of cavity 1, respectively. The ability of F771A to form a somewhat unstable and salt sensitive ternary complex strongly supports this notion.

The existence of another cavity, although less obvious (cavity 2, Figure 6), formed mainly between the O- and O1-helices may also be functionally significant. The C-terminal end of the N-helix and a portion of the O2-helix also participate in the formation of this cavity. This cavity is more obvious in the "closed" ternary complex due to rotation of the O-helix toward the palm subdomain. Several residues conserved between KF and KlenTaq are present in this cavity. These residues are I779, L777, Y787, Y791, and F792. Because of their hydrophobic nature, these residues are likely to maintain the structural integrity of this region. It is interesting to point out that the helix−hinge−helix motif resembling the O-, O1-, and O2-helices has been identified in T4 RNase H, *E. coli* endonuclease III, and Mrf-2 (a transcription factor) (*51*). This motif of the above-mentioned proteins has been implicated in the binding of gapped DNA substrates. Therefore, it is likely that in pol I, this region may also recognize and bind to the duplex region ahead of the primer terminus in the gapped DNA.

In conclusion, we have identified F771 of pol I as the single-stranded template interacting site and found that it helps in providing the stability to the pre-polymerase ternary complex. The modeling studies suggest that the region containing F771 may provide directionality and some stability to the template overhang.

ACKNOWLEDGMENT

We are grateful to Myron Crawford and Ken Williams of the William M. Keck Foundation Biotechnology Resource laboratory at Yale University for radiochemical and amino acid sequencing of the cross-linked peptide. We are also thankful to Dr. Robert J. Donneley of the Molecular Resource Facility at the New Jersey Medical School of the University of Medicine and Dentistry of New Jersey for the confirmation of the peptide sequence. We thank Dr. Harold Calvin for critically reading the manuscript and Dr. Rajiv Gangurde for general counsel, particularly in the ternary complex assay.

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BI026699Y