Significance of the O-Helix Residues of *Escherichia coli* DNA Polymerase I in DNA Synthesis: Dynamics of the dNTP Binding Pocket†

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ABSTRACT: In order to identify functionally important residues in the O and O1 helices of *Escherichia coli* DNA polymerase I, we mutated 9 residues of this region to alanine. The alanine substitutions result in moderate to severe effects on the polymerase activity of the individual mutant enzymes. Severe loss of activity is associated with R754A, K758A, F762A, and Y766A. However, the loss of polymerase activity with different template primers exhibited a rather unique pattern implying differential participation of the individual residue in the synthesis directed by poly(rA), poly(dA), and poly(dC) templates. The ability of all mutants to form E−DNA binary complex was found to be unaffected with the exception of Y766A and F771A, where signifcant reduction in the cross-linking of both the template and the primer strand was noted. Most interestingly, the catalytic activity of all inactive mutant enzymes, with the exception of K758A, could be restored by substituting Mn^{2+} in place of Mg^{2+} as a divalent cation. Based on these results and associated changes in the kinetic parameters and other properties of the individual mutant enzyme, we conclude the following: (a) Tyr 766 and Phe 771 are either involved in the binding of template-primer or are in the vicinity of the DNA binding track. (b) Residues Arg 754, Lys 758, Phe 762, and Tyr 766 appear to be required for the binding of Mg·dTTP, while only Arg 754 and Lys 758 are utilized in the polymerization of Mn·dTTP. (c) In the polymerization of dGTP, only Lys 758 appears essential regardless of the type of divalent cation. (d) Phe 762 participates only in the binding of Mg·dTTP. Finally, (e) based on the analysis of the time course of nucleotide incorporation, processivity, and pyrophosphorolysis reaction, we suggest that Lys 758 is probably involved in a conformational change of the ternary complexes preceding and following the chemical step. In summary, our results suggest that the formation of the dNTP binding pocket is a dynamic process which requires the participation of different residues depending on the type of dNTP and the divalent cation.

Escherichia coli DNA polymerase I is a monomeric protein (103 kDa) with three distinct enzyme activities, viz., DNA polymerase 3′−5′ exonuclease and 5′−3′ exonuclease (Kornberg & Baker, 1992). The proteolytically derived large fragment (Klenow) of this enzyme possessing polymerase and 3′−5′ exonuclease activities has served as an ideal model to understand the molecular mechanism of template dependent DNA synthesis. The 3-D crystal structure of the Klenow fragment has shown that the molecule is folded into two distinct domains, a 200-amino acid long, 3′→5′ exonuclease domain and a 400-residue polymerase domain separated by about 35 Å (Ollis et al., 1985; Beese et al., 1993). The polymerase domain containing the large cleft is further folded into three subdomains which have been referred to as the palm, the fingers, and the thumb motifs (Kohlstaedt et al., 1992). In the 3-D crystal structure, helices M, N, O, and P and part of the Q-helix constitute the finger motif which seems to be a constituent of the catalytic cleft. A number of amino acid residues in this motif have been found to play some role in the catalysis of DNA synthesis. For example, pyridoxylation of Lys 758 in the O-helix was shown to result in a complete loss of E−dNTP binary complex formation as well as near complete loss of the polymerase activity (Basu et al., 1987). Site directed mutagenesis of this residue provided further insight regarding the role of Lys 758 in the overall catalytic process and especially in copying as well as in translocating along the dA sequence in the template strand (Pandey et al., 1994a). Chemical modification of Arg 841 residing in the Q-helix was found to result in the loss of DNA binding and catalysis (Mohan et al., 1988). Site directed mutagenesis of Arg 841 and Asn 845 has been reported to cause a 10-fold increase in $K_{d(DNA)}$ and $K_{m(dNTP)}$, respectively, implying the participation of these amino acid residues in the binding of template-primer and dNTP (Polesky et al., 1990, 1992). As regards Tyr 766, it has been shown to be involved in the binding of the primer terminus, since the terminal nucleotide of the primer containing the photoaffinity azido group cross-linked specifically to this residue (Catalano et al., 1990). A Tyr 766 Ser mutant has also been shown to be an error-prone enzyme (Carrol et al., 1991). In addition, this mutant (Y766S)[1] was found to be selectively defective in reading dA templates, while its activity with other hetero- and homopolymeric template-primers seemed only slightly affected (Desai et al., 1994). Model building of E−TP binary complex has suggested possible involvement of the finger subdomain (O-helix) in the binding of the template strand (Besse et al., 1993). Using a self-annealing DNA template-primer, as the affinity label, we reported that the covalent cross-linking of this DNA to

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the Klenow fragment is in its polymerase mode. By means of peptide mapping of the labeled enzyme, we then showed that a peptide spanning residues 759−775, which constitutes part of the O- and O1-helices, contains the binding domain for the template-primer (Pandey et al., 1994b). Therefore, residues in the O- and O1- helix region appear to play a significant role in all aspects of the polymerase reaction. In order to clarify the contribution of the individual residues of this region, we carried out site directed mutagenesis of 9 residues, namely, Glu 752, Arg 754, Arg 755, Lys 758, Phe 762, Ile 765, Tyr 766, Ser 769, and Phe 771. The individual mutant enzymes were then characterized with regard to their biochemical properties. Results showed that mutations at 4 of the 9 sites caused a significant defect in the catalytic activity, but the extent of defect differed with respect to synthesis directed by different template-primers and the type of divalent cation used. While this work was in progress, Joyce and colleagues reported similar mutagenesis studies pertaining to these four residues and demonstrated their catalytic importance for the polymerase function (Astatke et al., 1995). Our results are in general agreement with their report, but we extend these observations further to clarify the roles played by these four residues in the overall catalytic process. Our results strongly suggest that all four residues participate in the formation of an Mg·dTTP binding pocket, while binding of Mg·dGTP requires participation of only Arg 754 and Lys 758. Substitution of Mn^{2+} in place of Mg^{2+} restored the activity of all the mutants with the exception of Lys 758 Ala. These results imply that the divalent cations are capable of modulating the structural components of the dNTP binding pockets. The significance of these observations in the light of a mechanistic scheme for the progression of polymerase reaction is also discussed.

MATERIALS AND METHODS

Materials. *E. coli* DNA polymerase I (Klenow fragment) was purified from an overproducing exonuclease deficient strain (Derbyshire et al., 1991) generously provided by Catherine Joyce of Yale University. Wild type Klenow fragment was expressed and purified from an overproducing clone (PET-3a-K) in *E. coli* BL-21 (DE-3) as described before (Pandey et al., 1993; Kaushik et al., 1993). Restriction endonucleases and DNA modifying enzymes were from Promega or Boehringer Mannheim. Homopolymeric template-primers and HPLC purified dNTPs were obtained from Pharmacia, while tritiated and ^{32}P labeled dNTPs and ATP were the products of Dupont-New England Nuclear Inc. Sequenase and other reagents were from US Biochemicals. Synthetic self-annealing template primer (see Chart 1) were obtained from Midland Certified Reagent Co. The synthetic template-primers used in the assay were prepared by annealing equimolar amount of template and primer at 20 μM concentrations. *In vitro* mutagen Kit for mutagenesis was obtained from Bio-Rad. All other reagents were of the highest available purity grade and purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad.

Chart 1: Oligomeric Template-Primers

37 meric self-annealing TP

CACGCAGTCTTCXCC $^{-3'}$

TCACGTCAGAAGAGGATCCCTC $^{-5'}$

X = bromo-dU

47/18 mer TP

5'-CTTCCATTCACACACTGC-3'

3'-GAAGGTAAGTGTGTGACGATGTCTGACCTTGTTTTTGTGACATTGAG-5'

26/18 mer TP

5'-CCGGCCAAAAAAAAAAAAA-3'

3'-GGCCGGTTXTTXTTXTTXTTXTTXTT-5' X = bromo-dU

M13mp19 template annealed with 21 mer primer

5'-GGATCACCAGCAATATTCCAA-3'

3'-CCTAGTGGTCGTTATAAGGTTTCATCGTACTGTTTTTAGAATCTCGCAAAATCT-5'

In Vitro Mutagenesis and Construction of Expression Plasmid. Site directed mutagenesis was performed as described by Kunkel at el. (1987). Mutations at residues E752, R754, R755, K758, F762, I765, Y766, S769, and F771 were introduced on a uracil containing M13mp19 template carrying the 1.1 kilobase *Sac*1 and *Hin*dIII fragment of the wild type pET-3a-K (Kaushik et al., 1993; Pandey et al., 1993) encoding the polymerase domain of pol I. Phosphorylation of the mutagenic primers annealing, extension, and ligation was carried out in accordance with the manufacturer's protocol supplied with the Mutagen M13 *in vitro* mutagenesis kit (Bio-Rad). After ascertaining the mutation in M13, the 1.1 kilobase mutated fragment was subcloned from M13mp19 into the wild type pET-3a-K cassette as well as the exonuclease deficient pET-3a-K cassette to obtain mutants with and without 3′→5′ exonuclease activities, respectively. The construction of the exonuclease deficient pET-3a-K clone was carried out by replacing Asp 424 to Ala 424 (D424A) by a two step polymerase chain reaction (PCR) using the megaprimer technique (Pandey et al., 1993). The 0.7 kilobase *Nde*I and *Sac*I digest of the 2nd step PCR product carrying the D424A mutation was cloned in the wild type pET-3a-K cassette. After the initial screening for the presence of the insert, the mutation was confirmed by double-stranded dideoxy DNA sequencing of the mutated region (Sanger, 1977). Each of the mutant clones was introduced into *E. coli* BL-21 (DE3) for induction by isopropyl 1-thio-β-D-galactopyranoside (IPTG) and purified by ammonium sulfate fractionation and Biorex-70 column chromatography as described before (Pandey et al., 1994a). The protein concentrations were determined by Bradford colorimetric assay (Bradford, 1976) as well as by spectrophotometric measurement using $E_{278} = 6.32 \times 10^4\ M^{-1}\ cm^{-1}$ (Setlow et al., 1972).

Enzyme Assay. Polymerase activity was determined as described before (Basu & Modak, 1987) with slight modification. In brief, assays were carried out in a final volume of 100 μL containing 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 10 μg of bovine serum albumin, 100 mM KCl, 10 mM $MgCl_2$, 250 nM of synthetic homopolymeric template-primer, and 20 μM of corresponding [^{3}H]dNTP (1000 cpm/pmol).

[1] Abbreviations: A, D, E, F, I, K, Q, R, S and Y represent the single letter codes for Ala, Asp, Glu, Phe, Ile, Lys, Gln, Arg, Ser, and Tyr, respectively; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl β-thiogalactopyranoside; poly(rA)·$(dT)_{18}$; polyriboadenylic acid annealed with $(oligodeoxythymidylic\ acid)_{18}$; poly(dA)·$(dT)_{18}$, polydeoxyriboadenylic acid annealed with $(oligodeoxythymidylic\ acid)_{18}$;poly(dC)·$(dG)_{18}$, polydeoxycytidylic acid annealed with $(oligodeoxyguanylic\ acid)_{18}$; dNTP, deoxyribonucleoside triphosphate; dATP, dGTP, dCTP, and dTTP represent nucleoside triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxythymidine, respectively; PP_i, pyrophosphate; PLP, pyridoxal 5′-phosphate; WT, wild type; TP, template-primer.

In some experiments, synthetic heteropolymeric template primer and all four dNTPs (20 μM each) along with 1 μCi of [α-^{32}P]dTTP or [α-^{32}P]dATP were used in the assay mixture. Incubations were carried out at 37 °C for 5−15 min, and reactions were terminated by the addition of 5 mL of ice cold trichloracetic acid containing 1 mM pyrophosphate. The reaction mixtures were filtered through Whatman glass fiber filter (GF-B), and radioactivity in the acid insoluble fraction was determined by scintillation counting. 3′→5′ exonuclease activity was measured either on double-stranded DNA (Pandey & Modak, 1988) or on single-stranded DNA substrates (Derbyshire et al., 1991).

Cross-Linking of dNTP and Template-Primer DNA to Enzyme. In order to examine if the mutational changes on the O- and O1-helices caused any perturbation in the dNTP binding function of the enzyme, the extent of UV mediated cross-linking of each of the mutant enzymes to substrate Mg·dNTP or Mn·dNTP (binary complex) was determined. For the cross-linking experiment, 1 μM mutant enzyme was incubated on ice for 10 min. with 2 μM [α-^{32}P]TTP (40 μCi/nmol) in a buffer containing 2 mM $MgCl_2$ (or 0.1 mM $MnCl_2$), 50 mM Tris-HCl, pH 7.8, and 2% glycerol. The reaction mixture was then exposed to UV irradiation in a Spectrolinker (Spectronic Corp.) at a dose of 300 mJ/cm^2. Measurement of covalent attachment of [^{32}P]TTP to the enzyme protein was performed by sodium dodecyl sulfate−polyacrylamide get electrophoresis (SDS−PAGE) followed by autoradiography and quantification either by densitometric scanning of the autoradiogram or by excising the radioactive bands from the gel and measuring the Cerenkov radiation associated with the gel pieces.

For cross-linking of DNA template-primer, we used a self-annealing 37 mer template-primer as well as a homopolymeric 26/18 mer template-primers, containing 5′-CCGGCC-3′ and 3′-GGCCGG-5′ as the clamping sequences in the primer and template strands, respectively, to assess the binding efficiencies (see Chart 1). The template strand contained photoreactive bromo-dU residues at desired position(s). The template or primer strand was labeled with ^{32}P at the 5′-position (Ausubel et al., 1987). The concentrations of TPs used in the cross-linking reactions were in the range of their K_d values observed for the wild type enzyme. The reaction mixture, in a final volume of 100 μL, contained 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 100 mM NaCl, 5% glycerol, 1.2 μM enzyme, 50 nM ^{32}P labeled 37 mer, or 100 nM 26/18 mer TP (15 000 cpm/pmol). The divalent cation, when used, was Mg^{2+} at 2 mM or Mn^{2+} at 0.1 mM. Reactions were incubated on ice for 10 min and were exposed to 254 nm UV irradiation at a dose of 300 mJ/cm^2 (Spectronic Corp.). For template-primers containing bromo-dUMP substitution in place of dTMP, the exposure of enzyme−(template-primer) to UV was affected by changing the conventional UV source (254 nm) to the one providing 312 nm UV in the same apparatus. The irradiation was carried out for 3 min at 10 cm distance from the UV source. The TP cross-linked enzyme species were analyzed by SDS−PAGE and the extent of cross-linking was quantitated as described above.

Catalytic Competence of the E−TP Covalent Complex. For this purpose, 0.05 nmol of each of the mutant and WT enzymes were cross-linked with unlabeled 37 mer self-annealing template-primer in a reaction mixture containing 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 50 mM NaCl, 2 mM $MgCl_2$, and 0.15 nmol of 37 mer DNA in a final volume of 50 μL. The nucleotidyltransferase activity of the E−TP covalent complex, on the immobilized TP, was then measured by incubating the irradiated mixture in the presence of 0.6 M NaCl and 2 μCi of [α-^{32}P]dTTP (0.02 nM) corresponding to the first template base. Incubations were carried out for 30 min at 25 °C, and reactions were terminated by the addition of 1% SDS containing 40 mM EDTA. An aliquot of the reaction mixture was subjected to SDS−polyacrylamide gel electrophoresis followed by autoradiography. The radioactivity associated with the E−TP covalent complex was determined by Cerenkov counting after excising the radioactive band from the gel.

Steady State Kinetics of Polymerization. Kinetic studies were carried out at 25 °C as described by Bryant et al. (1983) using homopolymeric or defined heteromeric DNA as the template-primer. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 100 μg/mL bovine serum albumin, 100 mM KCl, 2 mM $MgCl_2$, and variable concentrations of both template-primer and dNTP substrates. The concentration of the enzyme ranged from 2.5 to 100 nM, depending upon the specific activity of the mutant enzyme, and the incubations were at 25 °C for desired time. The reactions were initiated by the addition of Mg^{2+} (or Mn^{2+}) and terminated by the addition of 5% ice cold trichloroacetic acid at indicated time points. The trichloroacetic acid precipitable materials were collected on Whatman GF/B filter and counted for radioactivity in liquid scintillation counter as described before (Pandey et al., 1987).

Measurements of Pyrophosphorolysis Reaction Products. Pyrophosphorolysis activity of each of the O-helix mutants was estimated by analyzing the products of the reaction on denaturing polyacrylamide gels. The DNA substrate was prepared by annealing [5′-^{32}P](dT)$_{18}$ with poly(dA) at equimolar concentrations. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg BSA/mL, 5 mM $MgCl_2$, 2 nM poly(dA)·[5′-^{32}P](dT)$_{18}$ (10^6 Cerenkov cpm/pmol), 500 μM sodium pyrophosphate, and 4.5 nM mutant enzyme in a final volume of 6 μL. The reactions were carried out at 25 °C for 1 h and quenched with equal volume of Sanger's gel loading solution (Sanger et al., 1977). The samples were heated at 100 °C for 3 min and resolved by electrophoresis on a 16% denaturing polyacrylamide−urea gel. The labeled products were detected by autoradiography.

Processivity of DNA Synthesis. A single-stranded template of a recombinant M13mp19 annealed with 21 mer primer was used (see Chart 1) for a qualitative measurement of processivity of DNA synthesis by the mutant and wild type enzymes. 0.5 pmol of 21 mer primer was 5′ end-labeled with [^{32}P]ATP and annealed with 0.5 pmol of the recombinant M13m19 template. The appropriate enzyme was first incubated with the labeled TP in an incubation mixture containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2.5 nM primer termini, and 100 nM of the wild type enzyme or 400 nM of the mutant enzyme (R754A, K758A, F762A, Y766A) in a total volume of 2 μL. After incubation for 1 min at room temperature, the polymerase reaction was initiated by the addition of a 2 μL solution containing 200 μM dNTPs (50 μM each) and 20 μg of heparin as the trap in 50 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$. The reaction was allowed to continue at 25 °C, and 1.5 μL aliquots were removed at 1 and 5 min and mixed with 2 μL of stop solution

containing 0.5% SDS and 100 mM EDTA. Products formed with the individual enzyme were analyzed on a denaturing 10% polyacrylamide gel containing 7 M urea followed by autoradiography of the gel.

In order to confirm the specific pattern of stalling during catalysis of DNA synthesis by some mutant enzymes (i.e., strong stops in terms of certain sequence context), a heteromeric 47/18 mer DNA template-primer and homopolymeric poly(dA)·$(dT)_{18}$ were also used in place of M13 DNA in the processivity experiments.

RESULTS

Construction and Purification of Mutant Enzymes. Ten site directed mutants of selected residues at αO, αO-αO_1, and αO_1 finger subdomains of the Klenow fragment carrying single point mutation were constructed and expressed in *E. coli* as described before (Pandey et al., 1994a; Desai et al., 1994). The functional side chains of the selected αOαO_1 residues were replaced with alanine so that the observed impairment, if any, in the mutant enzyme could be correlated to the functional importance of the original residue in the polymerase reaction. The mutants E752A, E752Q, R754A, R755A, K758A, F762A, I765A, and Y766A represent the αO helix, while S769T and F771A are from the αO-αO_1 and αO_1 regions. The purified enzyme preparations were found to be homogeneous with an estimated purity of greater than 95%. The levels of expression, solubility, and yield as well as the chromatographic characteristics of all the mutant proteins were identical with that of wild type enzyme. All the mutants were exonuclease deficient and showed a heat inactivation pattern identical to the wild type KF exo (results not shown).

Specific Activities of the Mutant Enzymes on Different Template-Primers. Our earlier observations with mutants of two members of the O-helix, K758A and Y766S, suggested a template specific translocation defect in the mutant enzymes upon encountering the rA or dA nucleotides in the template strand (Pandey et al., 1994a; Desai et al., 1994). Consequently, a low k_{cat} with "A template" directed reaction was consistently noted with these mutants, when compared to poly(dC) directed reactions. In order to determine if other residues in these regions shared similar properties, we evaluated the polymerase activity of all the mutant enzymes with different template primers. The results shown in Table 1 provide a rather interesting pattern of template-primer utilization. The activity of E752A and E752Q mutants with all template-primers is not much changed as compared to the WT enzyme. However, all other mutant enzymes encompassing residues 754−771 clearly show a defect in using poly(rA) template, as their specific polymerase activities were found to be reduced to <1% to 45% compared to the WT enzyme. The most drastic reduction in the specific polymerase activity is seen with R754A, K758A, F762A, and Y766A mutants with both poly-(rA) and -(dA) templates. The mutants of R755, I765, S769, and F771 have nearly 80% of the wild type enzyme activity with poly(dA) template. With poly(dC) as a template, the major defect is noted only with K758A and R754A, while all the remaining mutant enzymes exhibited no change or small decreases in activity (Table 1).

Surprisingly, the severe impairment of catalytic activity observed with Mg^{2+} could be reversed when it was replaced by Mn^{2+} as the divalent cation. As shown in Table 1 (values shown in parentheses), with the exception of K758A, all mutants including R754A, F762A, and Y766A exhibited a substantial increase in specific activity by the change in the source of divalent cation. The catalytic activity of K758A was also found to be partially recovered in the presence of Mn^{2+}. Determination of the optimum concentrations of Mg^{2+} and Mn^{2+} for mutant enzymes and the wild type enzyme with individual template primer showed no change in metal optima (data not shown).

Table 1: Specific DNA Polymerase Activity of WT and Mutant Enzyme with Various Template Primers[a]

	percent of WT polymerase activity		
enzyme	poly(rA)·$(dT)_{18}$	poly(dA)·$(dT)_{18}$	poly(dC)·$(dT)_{18}$
WT	100	100	100
E752A	73	70	86
E752Q	77	81	88
R754A	1.2 (24)	2.3 (60)	21 (78)
R755A	25	88	95
K758A	0.6 (2.2)	0.7 (5.1)	1.4 (21)
F762A	1.1 (32)	11 (61)	72 (102)
I765A	37	85	94
Y766A	1.2 (11)	2.4 (72)	78 (103)
S769T	47	78	82
F771A	45	86	91

[a] The specific polymerase activity of WT Klenow fragment and its mutant derivatives was determined with the indicated template-primers as described in Materials and Methods. The values shown are the percent of polymerase activity with respect to WT enzyme. The 100% WT activity with poly(rA)·$(dT)_{18}$, poly(dA)·$(dT)_{18}$, and poly(dC)·$(dG)_{18}$ was 2.7×10^4, 2.1×10^4, and 1.1×10^4 units/mg of protein, respectively. One unit is defined as the amount of enzyme activity necessary to incorporate 10 nmol of dNMP into acid insoluble form in 30 min at 37 °C. The values shown in parentheses were obtained in the presence of Mn^{2+} as the divalent cation.

Kinetic Parameters of the Mutant Enzymes. In order to probe the catalytic role of the individual member of the O-helix, we determined the kinetic parameters of all the mutant enzymes in the presence of Mn^{2+} and Mg^{2+} using homopolymeric poly(dA)·$(dT)_{15}$ and poly(dC)·$(dG)_{15}$ as the template primers. The results are summarized in Tables 2 and 3. As seen in the initial specific activity determination (Table 1), only the R754A, K758A, F762A, and Y766A mutants exhibited maximal decrease in the presence of Mg^{2+}; the catalytic efficiency (k_{cat}/K_m) was reduced 6000-fold for K758A mutant, and nearly 200−300-fold for R754A, F762A, and Y766A mutants (Table 2). The changes in affinity of dNTP substrate for these mutants were rather mixed. The mutation at 754 (R754A) and 762 (F762A) positions reduced the affinity (increase in K_m) for Mg·dTTP by 40−50-fold, but had only slight effect on the affinity for Mg·dGTP substrate. Interestingly, the affinity for Mg·dTTP was only marginally affected by mutation at 758 position. All other mutant enzymes with the exception of K758A showed no significant change in the K_m for Mg·dGTP utilization. In fact, some mutant enzymes, e.g., R755A, I765A, and Y766A, showed apparent increase (3−7-fold) in their affinity (low K_m) for Mg·dGTP with poly(dC)·$(dG)_{15}$ template-primer.

In contrast to the above results, the polymerase activity of all the O-helix mutants with poly(dA)·$(dT)_{18}$, except K758A, was enhanced when Mg^{2+} was replaced with Mn^{2+}. As shown in Table 3, the k_{cat} of polymerization reaction with poly(dA)·$(dT)_{18}$ of the three mutants (R754A, F762A, and Y766A) was significantly restored by Mn^{2+}. Surprisingly,

Table 2: Kinetic Constants for the Polymerase Reaction Catalyzed by O-Helix Mutants and WT Enzyme with Mg·dNTP as Substrates[a]

	poly(dA)·(dT)$_{18}$				poly(dC)·(dG)$_{18}$			
enzyme	$K_{m(dTTP)}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	ratio, mutant/WT	$K_{m(dGTP)}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	ratio, mutant/WT
WT	3.6	8.5	2.4×10^6		2.7	3.90	1.4×10^6	
E752A	2.0	5.2	2.6×10^6	1	0.5	0.50	1.0×10^6	7×10^{-1}
R754A*	55	0.45	8.2×10^3	3.4×10^{-3}	8.3	0.25	3.0×10^4	2×10^{-2}
R755A	9.0	3.50	3.9×10^5	1.6×10^{-1}	1.0	1.01	1.1×10^6	7.8×10^{-1}
K758A*	6.5	0.0024	3.7×10^2	1.6×10^{-4}	15.6	0.13	8.3×10^3	5×10^{-3}
F762A*	65	0.52	8.0×10^3	3.3×10^{-3}	6.3	0.32	5.2×10^4	4×10^{-2}
I765A	4.6	3.3	7.2×10^5	3×10^{-1}	0.4	0.77	1.9×10^6	1.35
Y766A*	8.1	0.11	1.3×10^4	5.6×10^{-3}	0.5	0.32	6.4 × 105	4.5×10^{-1}
Y766S	3.0	0.26	8.6×10^4	3.6×10^{-2}	1.2	2.00	1.6×10^6	1.14
S769T	5.7	4.96	8.7×10^5	3.6×10^{-1}	1.5	0.53	3.5×10^5	2.5×10^{-1}
F771A	2.0	2.55	1.3×10^6	5.4×10^{-1}	4.0	0.92	2.3×10^5	1.6×10^{-1}

[a] The steady-state kinetic parameters for the WT Klenow fragment and its mutant derivatives were measured with indicated template-primers and corresponding dNTP substrate as described in the Materials and Methods. Mutant enzymes marked with asterisk (*) exhibited maximal change in the kinetic parameters.

Table 3: Effect of Mn^{2+} on the Kinetic Constants of Some of the O-Helix Mutants[a]

	poly(dA)·(dT)$_{18}$			poly(dC)·(dG)$_{18}$		
enzyme	$K_{m(Mn\cdot dTTP)}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$K_{(Mn\cdot dGTP)}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
WT	1.4	1.45	1.0×10^6	1.5	0.63	4.2×10^5
R754A	38	1.60	4.2×10^4	7.4	1.7	2.3×10^5
K758A	7.8	0.03	3.8×10^3	5.4	0.41	7.6×10^4
F762A	8.8	0.45	5.1×10^4	3.6	1.46	4.0×10^5
Y766A	5.7	1.06	1.9×10^5	5.0	0.3	5.8×10^4

[a] The steady-state kinetic parameters for the WT Klenow fragment and the indicated mutant derivatives were measured with two different template-primers and corresponding dNTP substrate in the presence of Mn^{2+} as described in the Materials and Methods.

among these mutant enzymes, only R754A exhibited reduced affinity for Mn·dTTP substrate (25-fold increase in K_m). Interestingly, F762A mutant, which exhibited a 50-fold reduction in the affinity for Mg·dTTP substrate, did not show any significant change in its affinity for Mn·dTTP. These observations suggest that R754 may be selectively involved in the binding of dTTP in the presence of either Mg^{2+} or Mn^{2+}, while F762 may be required for the formation of a binding pocket for dTTP only with Mg^{2+} as the divalent cation. With regard to Y766A, no significant change in the K_m for dTTP or dGTP with either Mg^{2+} or Mn^{2+} was apparent, suggesting thereby that this mutational change does not directly affect substrate dNTP binding.

Ability of Mutant Enzymes To Form E−dNTP and E−TP Binary Complexes. Since different mutant enzymes exhibited either significant or little loss of catalytic activity depending upon the divalent cation used in the assay mixture, it was important to determine if the loss of activity is related to their ability/inability to form either E−dNTP or E−TP complexes. Results shown in Figure 1 (panel A) indicate nearly complete loss of E−Mg·dNTP binary complex formation with R754A, K758A, and F762A mutants, while all other mutants showed nearly identical dTTP cross-linking compared to the WT enzyme. Surprisingly, a similar loss of E−Mn·dTTP binary complex formation was also seen with these mutants (Figure 1, panel B).

To determine the ability of αO-αO_1 mutants to form E−(template-primer) binary complex, two different template primers, a self-annealing 37 mer TP containing a single bromo-dU in the duplex region and a 26/18 oligo pair with five bromo-dU in the template strand (see Chart 1), were employed. Results of cross-linking of 37 mer self-annealing DNA as well as 26/18 mer DNA to all mutant enzymes are

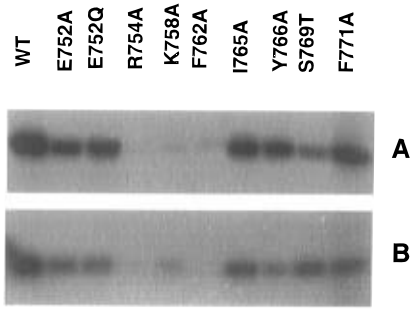

FIGURE 1: Ability of αO-αO_1 mutant enzymes to form enzyme−dNTP binary complex. The binding of dNTP substrate to various mutant enzymes was evaluated by UV mediated cross-linking in a standard irradiation mixture containing 2 μM [α-^{32}P]TTP and 1 μM enzyme protein in the presence of either Mg^{2+} (panel A) or Mn^{2+} (panel B). Cross-linked proteins were resolved by SDS−PAGE followed by autoradiography as described in the text.

shown in Table 4. All mutant enzymes exhibited nearly the same extent of cross-linking to both the template primers, except for Y766A and F771A mutants, which exhibited about 40−60% reduction in cross-linking. These results strongly suggest that the mutation of individual residues along the major span of O- and O_1-helices does not significantly affect the binding of template primer.

Catalytic Competence of Enzyme−DNA Covalent Complexes and the Effect of Mg^{2+} and Mn^{2+} on the Nucleotidyltransferase Activity. We have earlier shown that the wild type Klenow fragment covalently linked to template-primer, in its polymerase mode, can catalyze an addition of a single nucleotide onto the 3′-OH terminus of the immobilized template-primer (Pandey et al., 1994b). Since all mutant enzymes showed an ability to cross-link to 37 mer self-annealing template-primer in the absence of divalent cation,

Table 4: Photo-Cross-Linking of 37 mer Self-Annealing Template-Primer and 26/18 mer to WT Klenow Fragment and Its Mutant Derivatives[a]

enzyme	cross-linking to 37 mer self-annealing TP (% of WT enzyme)	cross-linking to 26/18 mer TP (% of WT enzyme)
WT Klenow	100	100
R754A	89	91
R758A	93	88
F762A	90	112
Y766A	44	40
F771A	51	60
other	100	100

[a] Photoaffinity cross-linking of 5′-^{32}P-labeled 37 mer self-annealing template-primer or 5′-^{32}P-labeled 26 mer template annealed with 18 mer primer (see Chart 1) to the Klenow fragment and its mutant derivatives was carried out as described in the text. The radioactive band containing protein−DNA complex was excised from the SDS−PAGE gel and counted for Cerenkov cpm. *Other enzymes include E752A, E752Q, R755A, I765A and S769T.

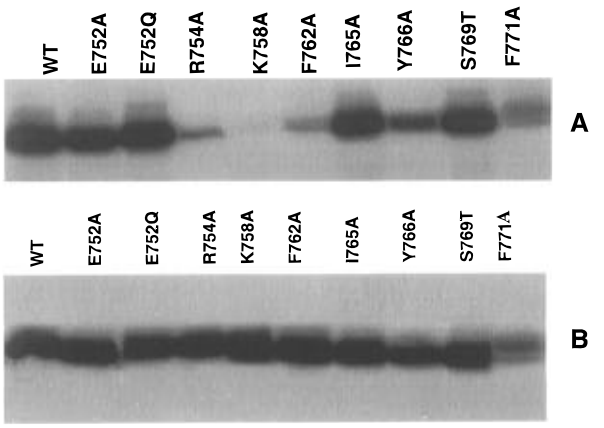

FIGURE 2: Catalytic competence of enzyme−TP covalent complexes of αO-αO$_1$ mutant enzymes: The WT enzyme and its αO-αO$_1$ mutant derivatives were cross-linked with unlabeled bromo-dU containing 37 meric self-annealing template primer in the absence of divalent cation as described in the Materials and Methods. Aliquots of the cross-linked complexes were incubated in a reaction mixture containing 50 mM Tris-HC1, pH 7.8, 1 mM DTT, 500 mM NaCl, and 2 μM [α-^{32}P]dTTP (corresponding to 1st template base) in a final volume of 50 μL. The reactions were initiated by the addition of either 5 mM $MgCl_2$ (panel A) or 0.5 mM $MnCl_2$ (panel B), and incubations were carried out at room temperature for 20 min. An aliquot of each reaction mixture was analyzed by SDS−PAGE and autoradiography as described before. The position of the radioactive band is approximately at 78 kDa position.

the effect of desired divalent cations (Mg^{2+} or Mn^{2+}) could be qualitatively assessed in the subsequent catalytic step by monitoring the nucleotidyltransferase activity of E−TP covalent complex. Results depicted in Figure 2 (panel A) clearly show that E−TP complexes of R754A, K758A, and F762A fail to catalyze nucleotide addition onto the immobilized template-primer, when Mg·dTTP was used as the substrate. In contrast, nucleotidyltransferase activity of the enzyme−(template-primer) covalent complexes of all the mutants was restored in the presence of Mn^{2+} (Figure 2B). It may be pointed out that the presence of divalent cation (Mg^{2+} or Mn^{2+}) has little effect on the extent of cross-linking of template-primer to the wild type as well as mutant enzymes (data not shown). The results of nucleotidyltransferase activity of enzyme−TP complexes of the mutant enzymes provide strong qualitative evidence to suggest that R754, K758, and F762 actively participate in the binding and/or turnover of Mg·dNTP, but may not be essential for binding of Mn·dNTP.

Rate Limiting Step of the Reaction. Since four O-helix mutant enzymes, namely, R754A, K758A, F762A, and Y766A, exhibited a substantial reduction in their catalytic efficiency, we carried out a time course of incorporation to identify whether the rate limiting step catalyzed by these mutants precedes or follows the phosphodiester bond formation step. Using poly(dA)·[5′-^{32}P](dT)$_{18}$ as the template-primer and excess of the individual mutant enzyme, the incorporation of dTMP was monitored at different time points and the reaction products were analyzed on polyacrylamide−urea gel. We expected that the determination of the time required to catalyze addition of 1st and 2nd nucleotides would indicate if the rate limiting step for these mutants is before or after the bond formation. For example, similar rate constants for the first and the second nucleotide incorporation would indicate that the reaction step affected by the mutation could be either the preceding or at the chemical step itself. If this is so, then under the conditions of processive synthesis, incorporation should be linear with time as has been observed with R754A and F762A mutants. Conversely, if the rate for the 2nd nucleotide incorporation is slower than that for the first nucleotide, then the reaction step affected by the mutation is likely to be after the chemical step. The incorporation pattern of such mutants would be nonlinear with time under the processive condition. Using [5′-^{32}P]dT 18 mer annealed with poly(dA) as the template-primer and an excess of the individual mutant enzyme, a time course of dTMP incorporation was performed and the reaction products were analyzed on polyacrylamide−urea gel. Figure 3A shows the time course of reaction products of R754A and K758A mutants as the representative of this type of experiments. As can be seen from the figure, the extension of (dT)$_{18}$ to 19, 20, and longer products by R754A mutant is linear with time, indicating identical incorporation rates for the first and the subsequent nucleotides. Similar results were obtained for F762A and Y766A mutants (data not shown), suggesting that the rate limiting step with these mutant enzymes occurs before or at the point of phosphodiester bond formation. In contrast, the extension of (dT)$_{18}$ to 19 mer product by K758A mutant was extremely slow, and the subsequent extensions to 19 and 20 mer products were further delayed and nonlinear with time, suggesting that both steps preceding and following the chemical step might have been affected by this mutant. This approach has previously been used to identify the reaction step affected by the carboxylate mutants (Polesky et al., 1991) and the K758A mutant (Pandey et al., 1994a) of the Klenow fragment and the R72A mutant of HIV-1 RT (Sarafianos et al., 1995).

Pyrophosphorolysis Activity of O-Helix Mutants. The above-described experiments pertain to the polymerase activity pattern of various mutant enzymes. Joyce and colleagues (Astatke et al., 1995) have earlier reported on the status of another catalytic activity, namely, the pyrophosphorolysis activity of these mutant enzymes. This activity may be considered as reversal of the polymerase reaction and therefore would require the participation of the same catalytic amino acid residues. Since polymerase activity of some of the O-helix mutants (R754A, K758A, F762A, and Y766A) was severely impaired, the effect of these mutations on the reverse reaction (pyrophosphorolysis) was determined

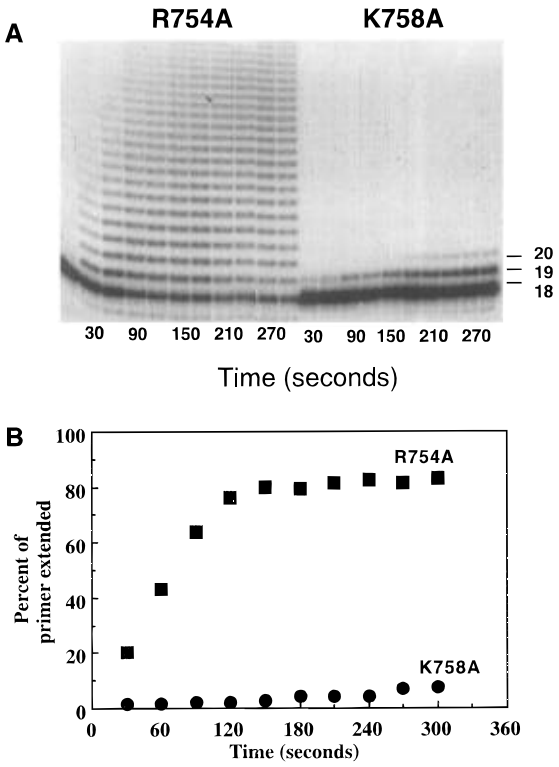

FIGURE 3: Time course of incorporation of dTTP by K758A and R754A mutant enzymes. (A) The 5′-^{32}P-labeled $(dT)_{18}$ annealed with poly(dA) was used to assess the time course of extension of $(dT)_{18}$ by R754A and K758A mutant enzymes. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg/mL bovine serum albumin, 100 mM KC1, 2 mM $MgCl_2$, 2.5 nM labeled template-primer, 200 μM dTTP, and 25 nM of individual mutant enzyme in a final volume of 60 μL. The reaction was initiated by the addition of metal ion at 25 °C. Aliquots (5 μL) were withdrawn at 30 s intervals and quenched with equal volume of Sanger's gel loading solution (Sanger et al., 1977). The reaction products were analyzed on a denaturing 16% polyacrylamide−urea gel followed by autoradiography on Kodak X-ray film. Note that the appearance of 19 mer product with K758A mutant enzyme occurs within 30 s, while 20 mer product could not be seen even after 150 s of incubation, suggesting a differential rate for the incorporation of the first and the subsequent nucleotides (Pandey et al., 1994a). (B) The amount of starting labeled material ($(dT)_{18}$) remaining unextended by R754A and K758A mutants in the above experiment (panel A) was determined by Cerenkov counting of the gel at each time point. The percent of total primer extended by each mutant was plotted as a function of time.

to assess the participation of the individual residues in this reaction by monitoring the degradation of [5′-^{32}P]dT_{18} in the presence of PP_i and poly(dA)·$(dT)_{18}$ (Astatke et al., 1995). The results shown in Figure 4 indicate that K758A is nearly devoid of pyrophosphorolysis activity, whereas R754A exhibited 20% of the WT activity. These results are qualitatively similar to those reported by Astatke et al. (1995). All other mutants including F762A, Y766A, and F771A were able to catalyze the reverse reaction as efficiently as the WT enzyme. These observations suggest that residues K758 and R754 may be sharing/interchanging their roles in dNTP and the PP_i binding in the forward and reverse reactions, respectively.

Effect of Pyridoxal Phosphate on the Pyrophosphorolysis Activity of the WT and Mutant Enzymes. We have earlier shown that pyridoxal phosphate (PLP) selectively reacts with K758 and inactivates the polymerase activity of the Klenow fragment (Basu & Modak, 1987). If K758 is indeed involved in some aspect of PP_i binding/release, then the pyrophosphorolysis reaction catalyzed by all the O-helix mutants should be highly sensitive to PLP modification. Our studies show that the WT enzyme and all the αO-αO_1 mutants are extremely sensitive to PLP treatment. The trace level of phophosphorolysis activity which was observed with K758A mutant remained unaffected by this reagent. Results with WT and K758A mutant enzymes are shown in Figure 5. This observation is consistent with the proposed role of K758 residue in PP_i binding/release reaction.

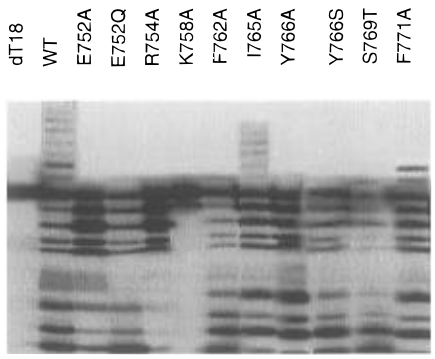

FIGURE 4: Ability of αO-αO_1 mutants of Klenow fragment to catalyze the pyrophosphorolysis. Poly(dA)·$(dT)_{18}$ was used as the template-primer for this experiment. 5′-^{32}P-labeled $(dT)_{18}$ annealed with the 36 mer dA template was incubated with the WT enzyme and its mutant derivatives for 60 min at 25 °C in the presence of 0.5 mM pyrophosphate as described in the Materials and Methods. Electrophoretic separation of the reaction products was carried out as described in Figure 3.

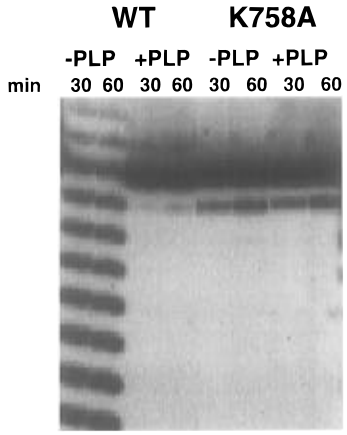

FIGURE 5: Effect of pyridoxal-5′-phosphate (PLP) on the pyrophosphorolysis activity of WT Klenow fragment and K758A mutant enzyme. WT Klenow fragment and K758A were subjected to PLP treatment followed by reduction with sodium borohydride as described before (Basu & Modak,, 1987). 30 nM PLP treated enzyme was examined for pyrophosphorolysis activity as described in Materials and Methods.

The Mode of DNA Synthesis by R754A, K758A, F762A, and Y766A Mutants. We have previously reported that K758A and Y766S mutants are defective in translocation when they encountered dA template base during DNA synthesis (Pandey et al., 1994a; Desai et al., 1994). To determine whether this template dependent translocation defect is unique to K758A and Y766S or is a common characteristic of other polymerase deficient mutants of this region, we determined the processivity of DNA synthesis by mutants of R754, K758, F762, and Y766 using M13mp19 single-stranded DNA template primed with 5′-^{32}P 21 mer primer. The template DNA contained dA bases at positions 3, 8, 17, 19, 20, 27, 28, 29, and 30 from the primer terminus (Chart 1). The wild type enzyme and its mutant derivatives

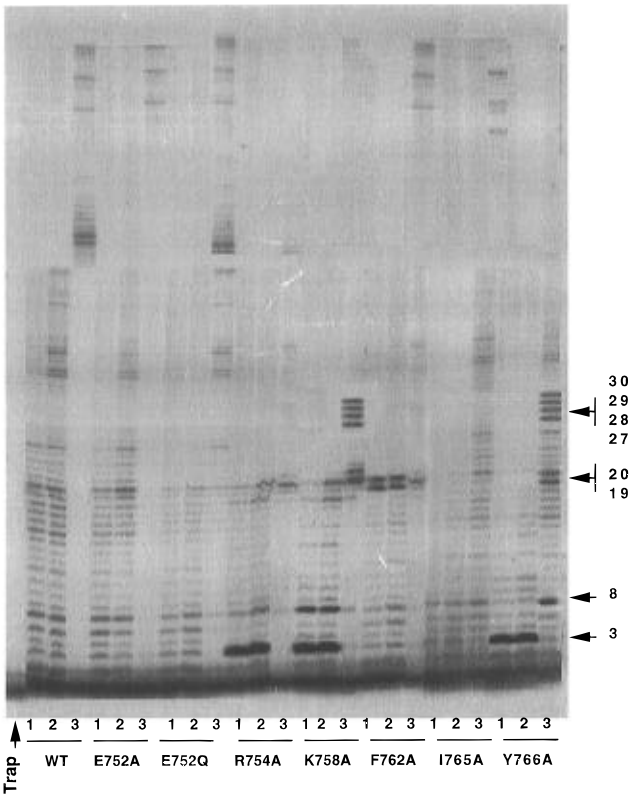

FIGURE 6: Processivity of DNA synthesis of αO-O_1 mutants. The 5′-^{32}P-labeled heteromeric 21 mer primer annealed with single-stranded M13mp19 (Chart 1) was used as the template-primer. Following the incubation of the individual enzyme with labeled TP, the reaction was initiated by the addition of four dNTPs and heparin as the trap (see Materials and Methods). The DNA synthesis observed with individual enzyme with or without heparin trap is shown in lanes 1−3. In lanes 1 and 2 of each set, heparin was added along with the dNTP substrates. The products generated under these conditions represent a single processive synthesis event during 1 min (lane 1) and 5 min (lane 2) of incubation. Lane 3 represents the DNA synthesis occurring on the labeled template-primer in the absence of heparin trap for 5 min. The extreme left lane marked as "Trap control" indicates the effectiveness of the trap added at the binding step (with the WT enzyme). Arrows on the right indicate the position of the dA base in the template.

were incubated with the labeled template-primer to allow the formation of enzyme−(template-primer) complex. The polymerization reactions were initiated by the addition of all four dNTPs together with heparin to trap the free and dissociated enzyme species. The results of this experiment are shown in Figure 6. Lanes 1 and 2 of each set are the processive synthesis (in the presence of trap) for 1 and 5 min, respectively, while lane 3 of each set represents both processive and nonprocessive synthesis (in the absence of trap). With the exception of R754A, K758A, and Y766A, all the mutants and WT enzyme, after incorporation of 1−100 nucleotides per processive cycle, dissociated from the labeled template-primer and effectively bound to the heparin trap. However, R754A, K758A, and Y766A appear to extend the primer until they encountered the dA template base at the 3rd position, resulting in large accumulation of 23 mer product within 60 s (Figure 6, see lane 1 of each of these three mutants). Further incubation of the reaction for 5 min does not significantly extend the accumulated 23 mer product. In the absence of the trap, the 21 mer labeled primer can be extended by these mutant enzymes until the next dA template base is encountered at positions 9, 17, 19, and 20 from the primer terminus as judged by the accumulation of the products at these positions (lane 3). It is interesting to note that F762A does not show similar stalling pattern on A templates; however, some accumulation of product at selective "A" positions is noted.

Additional confirmatory evidence to support stalling of polymerase defective mutant enzymes of this region upon encountering dA residue was obtained by replacing M13 system by heteromeric 47/18 mer and poly(dA)·$(dT)_{18}$ template-primers (data not shown). Results showed that all 3 mutant enzymes exhibit stalling at dA residue in the template region.

DISCUSSION

A number of previous studies have indicated that the O-helix region of the Klenow fragment contains catalytically important residues which are essential for the polymerase function of the enzyme. The first amino acid residue in this region to be identified as being catalytically crucial was Lys 758, which was implicated as the substrate dNTP binding site (Basu & Modak, 1987). Recent site directed mutagenesis of this residue, followed by extensive characterization, has expanded its role to include its participation in the reading of the dA region of the template strand as well as in the translocation reaction (Pandey et al., 1994a). Recent studies of photoaffinity labeling of the Klenow fragment with self-annealing DNA template-primer (Pandey et al., 1994b) have identified a part of the O-helix, beyond Lys 758 and extending up to residue 775, as the region involved in cross-linking to DNA template-primer. Thus, the primary sequence starting from 752 to 771 which contains the entire αO-αO_1 and their joining region (Joyce & Steitz, 1994) seems to participate in both the dNTP and template-primer binding functions of the polymerase domain. In order to clarify the participation of the individual residues of this region in the catalytic process, we have carried out site directed mutagenesis of 9 residues in this region, followed by characterization of the individual mutant enzyme.

While our investigation was in progress, Joyce and colleagues (Astatke et al., 1995) reported selective mutagenesis of residues R754, K758, F762, and Y766. These residues may be considered as true neighbors in the spatial context. A loss of catalytic activity was noted with all the four mutants. Our results generally confirm the catalytic importance of these four residues and provide additional information about these mutants which allows deductions concerning their role in the catalytic process. We would first like to point out rather interesting polymerase activity patterns that have emerged from our mutant studies. Our study encompassed the region from Glu 752 through Phe 771. The αO region is constituted by residue 752−766. Among these, Glu 752, Arg 754, Arg 755, Lys 758, Phe 662, and Tyr 766 are the amino acids with functional side chains. The fact that E752A, E752Q, and R755A exhibited little loss in the catalytic activity suggests that the side chains of these residues do not contribute toward the catalytic function. Similarly, relatively little loss of activity seen with mutants of other members of the O-helix and that of the O_1-helix, namely, S769 and F771, implies their noninvolvement in the catalytic process. Neverthless, the loss of catalytic activity measured with different template-primers as well as

divalent metal ions showed a differential degree of participation even among residues considered essential for catalytic activity. For example, under the same reaction conditions and with Mg·dTTP as an effective substrate, the four mutant enzymes exhibited severe loss of catalytic activity with poly(rA)·$(dT)_{18}$ template-primer, while the loss of activity with poly(dA)·$(dT)_{18}$ was relatively less severe especially with Y766A mutant (Table 2). However, under similar conditions, the catalytic efficiency of all the mutant enzymes, with the exception of K758A, was found to be either only moderately affected or not affected at all with poly(dC)·$(dG)_{18}$. Another interesting observation pertaining to these 4 mutants is the fact that catalytic activity with all the template primers could be recovered if Mg^{2+} in the reaction mixture was substituted by Mn^{2+}. The extent of recovery of K758A was rather limited (Table 3). These results strongly implicate different subregions of the O- and O_1-helix in copying the template nucleotides and/or formation of a nucleotide binding pocket and their dependence on the divalent cation.

The fact that cross-linking of template-primer to the mutants (with the exception of Y766A and F771A) was not significantly reduced, as compared to that of the WT enzyme, suggests that only Y766 and F771 either may be involved in the binding or are in the vicinity of the TP binding track. The results of our photoaffinity labeling studies with template-primer had indicated that the peptide belonging to this region contained the cross-linking sites for the template-primer (Pandey et al., 1994b). Furthermore, subtle differences in the mode of binding of specific template-primer are suggested by the pattern of template primer utilization by different mutants. The fact that all the effects related to dNTP utilization are restricted to four residues and that their inability to utilize dTTP is reversed by Mn^{2+} strongly implicates these residues (preceding Tyr 766) in dNTP binding and turnover.

Processivity experiments have demonstrated that, similar to K758A (Pandey et al., 1994a), Y766A and R754A mutants also exhibit a distinct pausing patten upon encountering a dA template base (Figure 6). F762A showed this restriction only when two or more dA bases occurred in the template. Some of these observations are at variance from those reported by Astatke et al. (1995), where pausing at dA template nucleotide was not noted. We have confirmed the dA pausing pattern with one of the mutant enzymes (R754A) generated in their lab. It may be that the template DNA used by these authors may have had dA bases too far from the primer terminus to detect a distinct pausing pattern. Since template nucleotide is a likely part of the active site in the polymerase reaction, the type of nucleotide base may influence the formation of the substrate binding pocket. Since Mg·dTTP is the substrate dNTP, the pausing at the dA residue may be attributed to the defect in the formation of the dNTP binding pocket with R754A, K758A, and Y766A. Some support to this contention is also found in the observation that substitution of Mg^{2+} by Mn^{2+} in the reaction mixture abolishes this pausing pattern of all mutant enzymes (data not shown).

The strongest evidence for the involvement of residues R754, K758, F762, and Y766 in the formation of appropriate dNTP binding pocket comes from the experiments where E−TP covalent complex is used as a source of enzyme. In the catalysis of the polymerase reaction, binding of dNTP is expected to follow the binding of template-primer to the enzyme. We previously demonstrated that the WT Klenow fragment covalently cross-linked with the DNA template-primer is catalytically competent and can efficiently incorporate a single nucleotide onto the immobilized template-primer (Pandey et al., 1994b). Therefore, the dNTP binding pocket in the E−TP covalent complex may be expected to represent the binding of dNTP in the ternary complex, and reduction in the catalytic ability of E−TP covalent complex may indicate impairment of dNTP binding/turnover. This system, therefore, provides an excellent means of qualitatively assessing the binding and turnover of a single dNTP as well as the effect of a divalent cation in this process, independent of template primer binding. Indeed, when the E−TP covalent complex of αO-αO_1 mutants were incubated with Mg·dTTP, the extent of nucleotide incorporation on the covalently bound template-primer was found to be significantly reduced only with those mutants which had shown reduced k_{cat} values (Figure 2A). With Y766A and F771A, the observed reduction in the incorporation of Mg·dTTP on the cross-linked TP was found to be proportional to the extent of their cross-linking to the template-primer. Therefore, these mutants do not appear to be defective in dNTP binding function.

Kinetic characterization of the four mutant enzymes displaying a defect in polymerase activity has shed further light on the binding of individual dNTP in these enzymes. A large increase in the K_m for Mg·dTTP for R754A and F762A mutants has been reported by Astatke et al. (1995). While our observations confirm these results, the magnitude of the increase is somewhat lower. Furthermore, we have not detected any significant change in the affinity for Mg·dGTP substrate by these enzymes. A possible explanation for the disparity in these results may be attributed to the presence of 100 mM KCl in our reaction mixture. Nevertheless, the high K_m values for Mg·dTTP do suggest the involvement of R754 and F762 residues in the binding of Mg·dTTP substrate. However, the most interesting and somewhat surprising result that emanated from these studies is the "near complete recovery" of k_{cat} of the mutant enzymes in the presence of Mn^{2+} as the divalent metal ion. The only exception to this was K758A; the severely impaired polymerase activity of this mutant enzyme was only partially reversed by Mn^{2+} ion. Although the R754A mutant completely recovered its k_{cat} in the presence of Mn^{2+}, reduction in its affinity for Mn·dTTP binding was nearly identical to that seen with Mg·dTTP, suggesting its involvement in the binding of dTTP substrate in the presence of either of the metal ions. The lack of any signifcant change in the K_m for Mn·dTTP by F762, in contrast to 50-fold increase observed with Mg·dTTP, was rather surprising. These results suggest that the coordination for Mn·dTTP substrate in the ternary complex may be distinctly different from that of Mg·dTTP and that the side chain of F762 may be too far to play a role in the binding of Mn·dTTP. As far as dGTP binding is concerned, the insignificant change in the $K_{m(dGTP)}$ by R754A and F762A in the presence of either Mg^{2+} or Mn^{2+} is also suggestive of their noninvolvement in the binding of dGTP. These results clearly suggest that the different O-helix residues may be involved in the formation of dTTP and dGTP binding pockets.

As far as the role of K758 in the catalytic process is concerned, it appears to be at a step beyond the initial binding

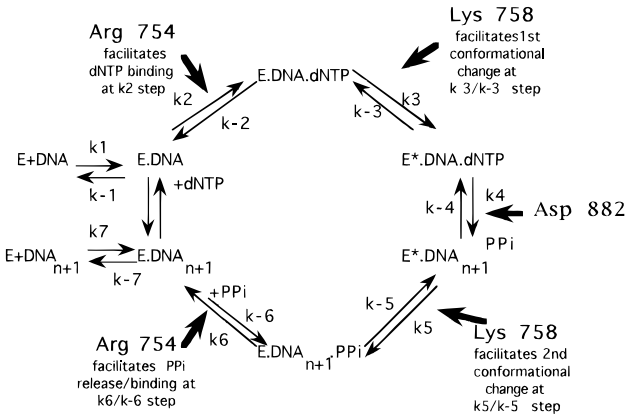

FIGURE 7: Postulated role of Arg 754 and Lys 758 in the kinetic scheme of DNA polymerization catalyzed by Klenow fragment. The above kinetic scheme for polymerase reaction has been adopted from Dahlberg and Benkovic (1991). In this scheme, we propose that the binding of dNTP in the E·DNA·dNTP ternary complex (k_2/k_{-2} steps) and that of PP_i (k_6/k_{-6} steps) are facilitated through Arg 754 as judged by high K_m for both dNTP and PP_i exhibited by R754A mutant. Participation of Lys 758 in this scheme is proposed to be at the k_3/k_{-3} and k_5/k_{-5}, where the respective ternary complexes in the forward and reverse reactions undergo conformational change preceding the chemical step (k_4/k_{-4}) which is probably catalyzed by Asp 882. This postulation is supported by extremely low k_{cat} of both the polymerase and pyrophosphorolysis activity displayed by K758A mutant with no concurrent loss in the affinity for dNTP and PP_i.

of dNTP since the K→A substitution at position 758 does not affect the affinity for dNTP substrate ($K_{m(dNTP)}$). This step could be a local conformational change in the enzyme molecule following the initial binding of dNTP to E−DNA binary complex or a conformational change in the bound dNTP itself along the glycosyl bond. We have previously reported a 3-fold difference in the rates of the incorporation of first nucleotide and the second nucleotide by K758A mutant enzyme (Pandey et al., 1994a). In the present investigation, we have noted that the incorporation of even the first nucleotide by this mutant enzyme is extremely slow and that the incorporation of second nucleotide is further reduced and not linear with time. These observations indicate that K758 may be required at catalytic steps that occur before and after the phosphodiester bond formation. We are suggesting the following scenario for the participation of both R754 and K758 in the catalytic process which consists of a number of distinct kinetic steps (see Figure 7). These steps are described in the kinetic scheme for polymerase reaction proposed by Dahlberg and Benkovic (1991). The two nonchemical steps proposed in this scheme are (i) the formation of E·DNA·dNTP ternary complex and (ii) subsequent conformational change of the ternary complex to a form (E*·DNA·dNTP) that is poised for catalysis. The kinetic constants obtained with mutants of R754 an K758 are consistent with the proposed kinetic scheme. Thus, in the polymerase reaction, R754 may be involved in the binding of dNTP in the first stage of E·DNA·dNTP ternary complex formation as judged by the unusually high K_m for dNTP exhibited by R754A mutant. K758 may then be postulated to play a role in the second step leading to the conformational change of E.·DNA·dNTP ternary complex to E*·DNA·dNTP complex. A drastic reduction in the k_{cat} (6000-fold) seen by K→A substitution at the 758 position without any significant change in the K_m for dNTP is the basis for this postulation. Similar to the "two step" binding mechanism for dNTP, the existence of a "two step" release mechanism for PP_i following the phosphodiester bond formation has also been suggested by Dahlberg and Benkovic (1991). These are (i) the conformational change of E*·DNA_{n+1}·PP_i species to E·DNA_{n+1}·PP_i ternary complex and (ii) the release of PP_i from the E·DNA_{n+1}·PP_i ternary complex. The assessment of the participation of various residues in the PP_i release steps was carried out using pyrophosphorolysis assays, a theroretical reversal of the polymerase reaction. It is reasonable to assume that the residues that participate in the forward reaction will also do so in the reverse reaction. Results with different mutant enzymes clearly show that mutants of only R754A and K758 are defective in catalyzing the pyrophosphorolysis reaction (Astatke et al., 1995). Furthermore, a significant increase in the $K_{m(PP_i)}$ for R754A but not for K758A mutant was also reported by these authors. Nevertheless, we estimate that the reduction in the catalytic activity of K758A was at least an order of magnitude higher than that seen with R754. Furthermore, absolute requirement for K758 in the pyrophosphorolysis reaction is also confirmed by sensitivity of R754A to PLP (Figure 5), a reagent which has been shown to react selectively with K758 (Basu & Modak, 1987). In light of these results, a proposition similar to the forward reaction (polymerase) may be conceived for the reverse reaction (pyrophosphorolysis) for the participation of K758 and R754. The initial PP_i binding may be suggested to occur through R754 as judged by high $K_{m(PP_i)}$ by its mutant R754A, while K758 may be implicated in the second step leading to the conformational change of E·DNA·PP_i ternary complex to E*·DNA·PP_i preceding the cleavage reaction. Furthermore, the observed low rate of incorporation of the first nucleotide and the reduction in the rate of addition of second nucleotide, especially with dA template base, by K758A (Pandey et al., 1994a) may now be explained on the basis of the inability of the mutant enzyme to undergo conformational change following the chemical step of the reaction.

In summary, the substrate dNTP binding pocket appears to have a rather complex geometry requiring participation of different residues, and our study has clarified some of these aspects with regard to dNTP binding. The fact that the divalent cation plays a major role in the process, as seen from the effect of Mg^{2+}/Mn^{2+} in the present study, also implies importance of the divalent cation coordinating residues, such as the members of the carboxylate triad (D882, E883, and D705). Preliminary examination of D882A, which is shown to be most crucial residue for the catalytic activity (Polesky et al., 1990, 1992), has revealed that D882A is deficient in pyrophosphorolysis activity (Kaushik et al., unpublished observation). Resolution of the ternary complex structure, and that of the enzyme−DNA binary complex (in polymerase mode), will permit further clarification of the dynamic process involved in the formation of individual dNTP binding pocket.

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