Participation of Active-Site Carboxylates of *Escherichia coli* DNA Polymerase I (Klenow Fragment) in the Formation of a Prepolymerase Ternary Complex†

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ABSTRACT: We have investigated the roles of four active-site carboxylates in the formation of a prepolymerase ternary complex of *Escherichia coli* DNA polymerase I (Klenow fragment), containing the template-primer and dNTP. The analysis of nine mutant enzymes with conserved and nonconserved substitutions of Asp^{705}, Glu^{710}, Asp^{882}, and Glu^{883} clearly shows that both catalytically essential aspartates, Asp^{705} and Asp^{882}, are required for the formation of a stable ternary complex. Of the two glutamates, only Glu^{710} is required for ternary complex formation, while Glu^{883} does not participate in this process. This investigation also reveals two interesting properties of the Klenow fragment with regard to enzyme−template-primer binary and enzyme−template-primer−dNTP ternary complex formation. These are (a) the significant resistance of enzyme−template-primer−dNTP ternary complexes to the addition of high salt or template-primer challenge and (b) the ability of the Klenow fragment to form ternary complexes in the presence of noncatalytic divalent cations such as Ca^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}.

Escherichia coli DNA polymerase I (pol I)[1] catalyzes DNA synthesis at a relatively high rate (∼50 nucleotides/s) (*1*), with a remarkably high fidelity (at an error rate of $\sim 10^{-6}$−10^{-8}) (*2*). This 109 kDa enzyme possesses three activities on a single polypeptide: the 5′−3′ polymerase, the 3′−5′ exonuclease, and the 5′−3′ exonuclease. The Klenow fragment (KF) of pol I is the C-terminal 68 kDa fragment which contains the polymerase and 3′−5′ exonuclease activities. The structure of the polymerase domain is reminiscent of a half-open right hand, with three subdomains called "palm", "fingers", and "thumb" (reviewed in ref *3*). The KF has been the subject of extensive biochemical studies, and continues to serve as a model system in the elucidation of the mechanism of DNA polymerization. To achieve its high level of accuracy, the enzyme relies upon two distinct levels of control during DNA synthesis: selection of a nucleotide triphosphate that forms a Watson−Crick base pair with the template base and excision of an incorrectly paired base by the 3′−5′ exonuclease activity of the enzyme. The first level of control, i.e., the selection of a correct nucleotide, is considered the most stringent of these controls, as it contributes maximally to the fidelity of the enzyme (*4*).

Pre-steady-state kinetic analyses have established that DNA and dNTP, the two substrates of the polymerase reaction, bind to the enzyme in an ordered manner. DNA binds first, forming an enzyme−DNA binary complex, which is followed by dNTP binding, resulting in the formation of an enzyme−DNA−dNTP ternary complex. The rate of dNTP incorporation is limited by a conformational change in this complex prior to the chemistry step. This conformational change causes the enzyme to lock down or capture the dNTP, slowing the rate of dissociation of the complex. Thus, nucleotide binding and the formation of a ternary complex poised for catalysis are two distinct steps in the reaction pathway and have been termed "open" and "closed" ternary complexes (reviewed in ref *5*). In the case of the KF, an open ternary complex is possible with any of the four dNTPs, since, at this stage, all four substrates bind to the enzyme−DNA binary complex with equal affinities (*6*); however, the open to closed transformation is brought about only by the correct dNTP, which is able to form a Watson−Crick base pair with the template nucleotide.

The theory of a rate-limiting conformational change (*5*), a kinetic entity proposed solely on the basis of solution studies (*1*, *4*, *7*), has acquired reasonable support from structural analyses of pol I family polymerases. Over the past few years, a number of crystal structures involving enzymes of the DNA pol I family have been determined (*8*−*15*), which has provided an opportunity to clarify the mechanism of substrate dNTP selection during DNA synthesis. A comparison of enzyme−DNA binary and enzyme−DNA−dNTP ternary complex structures from related polymerases of the pol I family, such as *Thermus aquaticus* DNA polymerase (*12*, *15*) and T7 DNA polymerase (*8*), strongly suggests a substrate-induced, conformational change in the fingers subdomain of the enzyme. The closed ternary complex differs from the open complex in the orientation

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[1] Abbreviations: pol I, *E. coli* DNA pol I; WT, wild type; KF, Klenow fragment; TP, annealed template-primer; dNTP, deoxynucleoside triphosphate; dATP, dTTP, dGTP, and dCTP, nucleoside triphosphates of deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine, respectively; BSA, bovine serum albumin. Protein mutations are described using the following convention: residue number preceded by the one-letter abbreviation for the wild-type amino acid and followed by the abbreviation for the substitution. Thus, E710A denotes the substitution of Glu with Ala at position 710.

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of the tip of the fingers subdomain which undergoes an inward rotation by ~40−46°, resulting in a "closing" of the polymerase active site (*12*, *15*). This substrate-induced motion seen in ternary complex structures has been likened to the rate-limiting conformational change proposed from the kinetic studies. Although the functional equivalence of the two remains unproven, it is assumed to be so (*8*, *15*, *16*).

Scott and colleagues have assessed ternary complex formation in HIV-RT using a gel-shift assay (*17*). In this assay, a prepolymerase enzyme−DNA−dNTP ternary complex is formed using a template-annealed, 3′-dideoxynucleotide-terminated primer, which inhibits the incorporation of the dNTP, resulting in a "dead-end complex". Thus, the assay permits an examination of the steps before chemistry and the events leading to the chemical step, i.e., DNA and dNTP binding, as well as conformational change. The gel-shift assay has also been successfully adapted for ternary complex formation studies with the KF (*18*, *19*) and its equivalent enzyme from *Mycobacterium tuberculosis* (*20*).

In this report, we have used the ternary complex formation assay to determine the individual participation of four active-site carboxylates of the KF of *E. coli* DNA pol I. In an earlier study (*21*), we demonstrated the requirement of a carboxylate triad at the active center, and found that the optimal activity of the enzyme depends on the presence of two such triads. Each triad comprises two active-site aspartates, Asp^{705} and Asp^{882}, which are essential for activity, and a vicinal glutamate at position 710 or 883. Using the ternary complex formation assay, we find that both essential aspartates are required for the formation of an enzyme−DNA−dNTP prepolymerase complex. Of the vicinal glutamates, only Glu^{710} participates in complex formation, while Glu^{883} is not required for this process.

EXPERIMENTAL PROCEDURES

Materials

PCR-grade dNTPs, rNTPs, ddNTPs, and DNA-modifying enzymes were from Boehringer Mannheim. Radiolabeled dNTPs were purchased from Perkin-Elmer. Synthetic oligomers were obtained from MWG-Biotech, and were purified by preparative electrophoresis on a 16% (w/v) polyacrylamide−urea gel. Radiolabeled oligomers were quantified using the DNA dipstick kit from Invitrogen. Amersham Biosciences' Hoefer SE 250 Mighty Small II Mini-Vertical Unit was used for electrophoresis.

Methods

WT and Mutant Enzymes. The WT KF and all the mutant derivatives used in this study have been described previously (*21*). All enzymes used in this investigation carry the D424A substitution which abolishes the 3′−5′ exonuclease activity (*22*, *23*).

Assay for Binary and Ternary Complexes. Formation of enzyme−template-primer (TP) binary and enzyme−TP−dNTP ternary complexes was assessed using a gel-shift assay where differential electrophoretic migration of enzyme-bound TP compared to that of free TP was used to assess the formation of both binary and ternary complexes, with some modification of previously described methods (*21*). To restrict the assay to the events preceding nucleotide incorporation, the 21-mer primer used in these studies has a 3′-dideoxyribose terminus. This primer was ^{32}P-labeled at the 5′-end, and annealed to its corresponding 33-mer template at a 5:1 (template:primer) ratio. Enzyme−TP binary complexes were formed in a 10 μL reaction mix containing varying concentrations of the WT KF or mutant enzyme mixed with the annealed TP (50−100 pM) in buffer containing 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.75 μM BSA, 0.05% (v/v) Nonidet P-40, and 10% glycerol. For ternary complex formation, 100 μM dNTP complementary to the template nucleotide was added to the same reaction mixture containing enzyme−TP binary complexes. Reaction mixtures were electrophoresed under nondenaturing conditions on an 8% (w/v) polyacrylamide gel (gel thickness, 0.75 mm; well volume, ~25 μL) prepared in 90 mM Tris-borate buffer (pH 8.2) and prerun for 1 h at 150 V and 4 °C. Electrophoresis was carried out with 45 mM Tris-borate buffer (pH 8.2) at 150 V for 1 h at 4 °C. Following electrophoresis, the gels were subjected to phosphorimaging, and the distribution of radiolabeled TP (free vs complexed) was determined using ImageQuant (Molecular Dynamics). The percent enzyme−TP binding was calculated by quantifying the amount of uncomplexed TP in each lane. Percent binding values were then used for the determination of $K_{D(DNA)}$ (concentration of the enzyme required to achieve half-maximal binding) by interpolation using nonlinear regression for one-site binding (hyperbola) using GraphPad Prism software.

Stability and Specificity of Enzyme−Substrate Complexes. The stability of enzyme−TP binary and enzyme−TP−dNTP ternary complexes was tested under two different conditions: (i) by challenging with excess TP and (ii) by increasing the ionic strength. The overall assay procedure remained the same, with the following modifications. For the TP challenge condition, binary or ternary complexes were formed by using 5′-^{32}P-labeled dideoxy-terminated 33/21-mer template-primer (50−100 pM) with 1 nM KF with or without 100 μM dGTP. These preformed binary or ternary complexes were then challenged with a 0−2000-fold molar excess of the same nonradioactive TP, and incubated for 5 min at 4 °C. Similarly, to assess the effect of increasing ionic strength, 25−800 mM NaCl was added to preformed binary and ternary complexes. The specificity of the closed ternary complex formation was examined with 50−100 pM 33/21-mer template-primer (5′-^{32}P-labeled and 3′-dideoxyribose) mixed with 1 nM KF and one of the four dNTPs (100 μM), or 100 μM ddGTP. To each mixture, 100 nM (~1000-fold molar excess) of unlabeled 33/21-mer TP was added, and the mixture was incubated for 5 min at 4 °C. In this analysis, an EDTA control lane was included to evaluate the requirement of Mg^{2+} for ternary complex formation. Besides Mg^{2+} being excluded from this reaction, 100 μM EDTA was added to chelate any divalent metal ions present in the enzyme and/or buffer solutions. The stability and specificity of the complexes under these conditions were determined by polyacrylamide gel electrophoresis under nondenaturing conditions as described above.

Effect of Divalent Cation. Since polymerase activity essentially requires the presence of metal ions (most likely providing dNTP in its metal chelate form), the effect of

various divalent cations on the formation of enzyme−TP binary and enzyme−TP−dNTP ternary complexes was assessed. Complexes were formed in a 10 μL reaction mix (similar to that described for binary and ternary complexes) using 50−100 pM 5′-^{32}P-labeled 33/21-mer (3′-dideoxyribose) TP with 1 nM KF. The reaction mix contained 100 μM EDTA, along with 10 mM Mg^{2+} or the divalent cation to be tested (at 0.5 mM). For ternary complexes, 100 μM dGTP was added to this reaction mix. The stability of the binary and ternary complexes was tested by adding NaCl to a final concentration of 0.5 M to preformed binary and ternary complexes. Electrophoresis of the reaction mixtures under nondenaturing conditions and the subsequent gel analysis were similar to those described above.

RESULTS

In this report, we have investigated the individual participation of four active-site carboxylates in the events prior to phosphodiester bond formation in the nucleotidyl-transfer reaction catalyzed by the Klenow fragment of *E. coli* DNA polymerase I. Since the focus of this study is on the polymerase active site, the WT enzyme and its mutant derivatives used in this investigation also contain the D424A substitution, which renders the WT or mutant enzyme exonuclease-deficient (*23*). Hence, the WT represents an enzyme with the D424A mutation, while the E710A species, for example, includes both E710A and D424A substitutions. All the enzymes used in this study have been purified and quantified under identical conditions, and mutant derivatives described herein are similar to the WT in terms of yield, purity (~95% pure, as judged by Coomassie blue staining of SDS−polyacrylamide gels), and solubility.

Binary and Ternary Complexes with the Klenow Fragment. Our initial assessment of the ability of WT and mutant enzymes to form enzyme−TP binary complexes with template-primer motifs containing a 3′-deoxy- or 2′,3′-dideoxyribose primer showed that the presence or absence of a 3′-OH at the primer terminus does not affect $K_{D(DNA)}$. This is in good agreement with an earlier report using the WT KF (*18*). Hence, $K_{D(DNA)}$ values obtained for template-primers with a dideoxy-terminated 3′-end can be safely used to assess the DNA binding affinity of the KF and its mutant derivatives.

Since enzyme−TP binary complex formation is a prerequisite for the binding of dNTP to produce a stable ternary complex, we assessed the effect of complementary dNTP (with respect to the template-nucleotide) on enzyme−TP binding. An increase in the level of enzyme−TP binding in the presence of dGTP, which is the next incoming nucleotide on the 33/21-mer TP used for this assay, was clearly noted (Figure 1). Using a low concentration of the radiolabeled TP (50−100 pM), our $K_{D(DNA)}$ results for KF are consistent with earlier reports for the same enzyme (*18*, *24*). The $K_{D(DNA)}$ value for enzyme−TP−dNTP ternary complexes was ~4-fold lower than that for enzyme−TP binary complexes. Similarly, on a 33/21-mer TP, where the template base was adenine, the corresponding complementary nucleotide, dTTP, produced a 10-fold decrease in the $K_{D(DNA)}$ value, further confirming the effect of the correct dNTP on enzyme−TP binding (data not shown). These results strongly indicate that

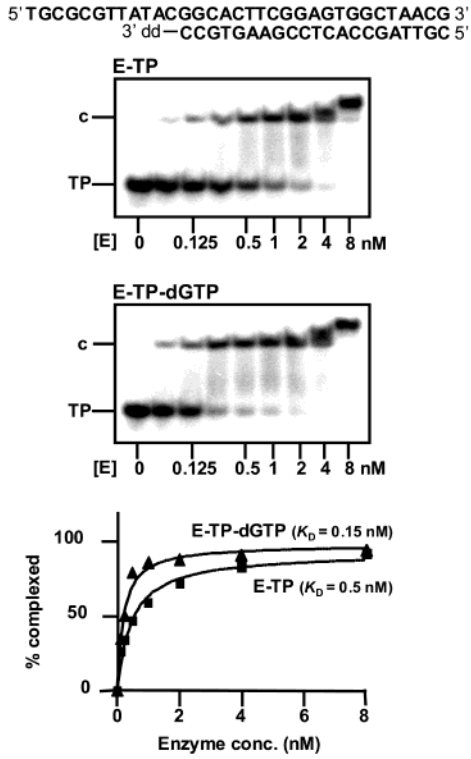

FIGURE 1: The presence of the correct substrate dNTP increases the extent of DNA−Klenow fragment binding. The 5′-^{32}P-labeled, 3′-dideoxyribose-containing 21-mer primer annealed to its corresponding 33-mer template was incubated with varying concentrations of the KF and electrophoresed under nondenaturing conditions as described in Experimental Procedures. The TP sequence is shown at the top. Autoradiographs of comparative TP binding in the presence and absence of substrate dGTP (center) show uncomplexed TP (marked TP) and enzyme−TP or enzyme−TP−dGTP complexes (marked c). The bottom panel is a plot of percent DNA complexed as a function of enzyme concentration, which was used for the determination of $K_{D(DNA)}$.

the increase in the affinity of the KF for TP resulted from the binding of dNTP (or the formation of a ternary complex).

Stability and Specificity of Enzyme−Substrate Complexes. Examination of the stability of enzyme−TP binary and enzyme−TP−dNTP ternary complexes upon the addition of competing TP or excess NaCl showed that enzyme−TP−dNTP ternary complexes were significantly more stable than enzyme−TP binary complexes. Enzyme−TP−dNTP complexes could be detected even in the presence of a 2000-fold molar excess of competing TP (nearly 40% of the original complex was retained). Similarly, these complexes were stable to the addition of NaCl concentrations of up to 1 M. On the other hand, preformed enzyme−TP binary complexes are readily dissociated with a 100−200-fold molar excess of TP, or with NaCl concentrations of >0.5 M (Figure 2). Clearly, ternary complexes are substantially more stable than binary complexes.

When noncomplementary dNTPs or the dideoxyribose analogue of the complementary dNTP was used in place of the complementary dNTP, enzyme−TP complexes exhibited sensitivity to excess TP (Figure 3) or to high ionic strength, implying that no stable ternary complexes could be formed with nonsubstrates. This result concurs with earlier reports by Romano and colleagues about the specificity of ternary complexes formed by the KF (*18*, *19*). Even with the correct

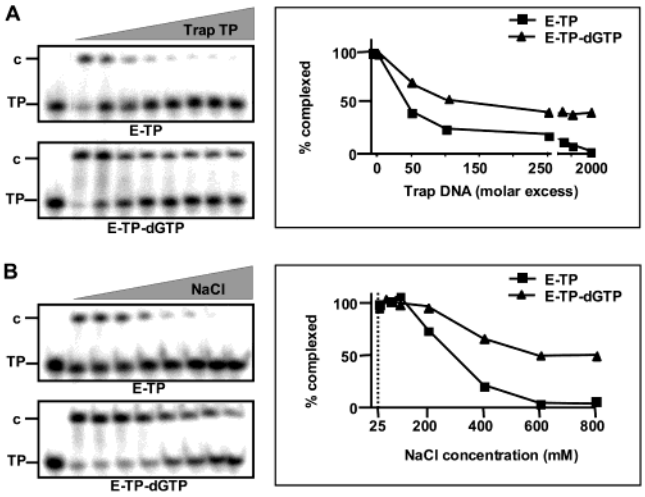

FIGURE 2: Enzyme−DNA−dNTP ternary complexes are more stable than enzyme−DNA binary complexes. The 5′-^{32}P-labeled, 3′-dideoxyribose-containing 21-mer primer annealed to its corresponding 33-mer template was incubated with 1 nM Klenow fragment with or without 100 μM dGTP (correct incoming substrate on this TP). The relative stabilities of preformed enzyme−TP binary complexes and enzyme−TP−dGTP ternary complexes were tested in the presence of increasing amounts of competing 33/21-mer TP (0−2000-fold molar excess over radiolabeled 33/21-mer) and increasing ionic strength (NaCl concentrations from 25 to 800 mM). Autoradiographs of binary and ternary complexes were used for measurement of the amount of complexed vs free TP as a function of the amount of competing TP (A) and NaCl (B).

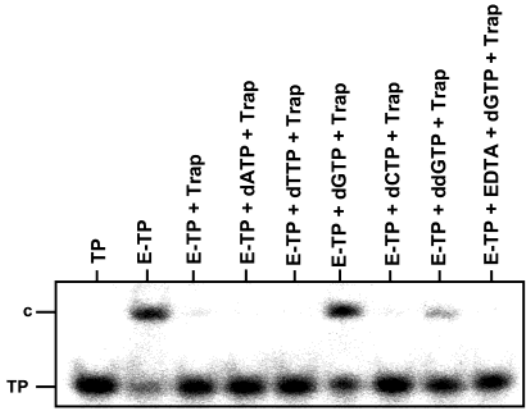

FIGURE 3: Klenow fragment forms a stable ternary complex only with the correct incoming nucleotide. Preformed enzyme−TP (5′-^{32}P-labeled, 3′-dideoxyribose-containing 21-mer primer annealed to its corresponding 33-mer template) binary complexes (50−100 pM TP and 1 nM KF) were competed with a 1000-fold molar excess TP (unlabeled 33/21-mer) in the presence of 100 μM correct (dGTP in this case) or incorrect substrate dNTPs, or 100 μM ddGTP, and electrophoresed under nondenaturing conditions as described in Experimental Procedures.

substrate dNTP, the presence of a divalent cation is necessary for complex formation, since inclusion of EDTA in the reaction mix prevented the formation of a stable ternary complex (Figure 3).

Role of Active-Site Carboxylates in Binary and Ternary Complex Formation. We have shown that in addition to two highly conserved aspartates, Asp^{705} and Asp^{882}, participation of either Glu^{710} or Glu^{883} is required for optimal polymerase activity (*21*). To evaluate the individual roles of these carboxylates in the formation of binary and ternary complexes, we examined nine mutant enzymes, including homologous (E710D, D882E, and E883D) and nonhomologous (D705A, E710A, D882A, and E883A) single substitutions of the carboxylates, and two double substitutions of the glutamates (E710A/E883A and E710D/E883D). The formation of a stable ternary complex was judged by two parameters: (i) an increase in the level of enzyme−TP binding in the presence of the correct dNTP and (ii) significantly increased resistance of the ternary complex to trap-TP competition as compared to that of an enzyme−TP binary complex formed under similar conditions.

Table 1: DNA Binding Affinity of the Klenow Fragment and Its Carboxylate Mutant Derivatives in the Presence and Absence of Substrate dGTP[a]

enzyme	$K_{D(DNA)}$[b] (nM)	$K_{D(DNA)}$ with dGTP[c] (nM)
WT	0.50 ± 0.2	0.15 ± 0.05[d]
D705A	0.63 ± 0.1	0.60 ± 0.04
E710A	1.8 ± 0.4	2.10 ± 0.1[d]
E710D	0.7 ± 0.3	0.18 ± 0.08
D882A	0.54 ± 0.2	0.3 ± 0.1
D882E	0.71 ± 0.2	0.33 ± 0.1
E883A	0.61 ± 0.3	0.16 ± 0.05
E883D	0.55 ± 0.2	0.14 ± 0.05
AA[e]	2.2 ± 0.6	2.3 ± 0.1
DD[f]	0.7 ± 0.2	0.16 ± 0.05

[a] Determined by the gel-shift assay using 50−100 pM 5′-^{32}P-labeled, 3′-dideoxyribose-containing 21-mer primer annealed to its corresponding 33-mer template with varying enzyme concentrations as described in Experimental Procedures. For each enzyme, 8−10 enzyme concentrations were chosen to bracket the expected $K_{D(DNA)}$ range. Values represent an average of three independent determinations. [b] We have previously assessed the DNA binding affinity of the KF and some of the carboxylate mutant enzymes (*21*), and $K_{D(DNA)}$ values reported here are ∼10-fold lower than in the earlier report. In the earlier study, the 5′-^{32}P-labeled, self-annealing 37-mer was used at a concentration of 3 nM, as opposed to the 50−100 pM input TP used in this study. We have noted that with a higher input TP level, $K_{D(DNA)}$ values change significantly. Nonetheless, irrespective of the TP concentration that is used, the relative binding affinities between the WT and the individual mutant enzymes remain unaltered. [c] Substrate dGTP, which is the next incoming nucleotide on the 33/21-mer template-primer, was added at a final concentration of 100 μM. [d] For WT and E710A enzymes, $K_{D(DNA)}$ values with all four dNTPs were 0.13 ± 0.05 nM and 1.8 ± 0.3 nM, respectively, and $K_{D(DNA)}$ values with three dNTPs (excluding dGTP) were 4 ± 0.7 nM and 1.9 ± 0.3 nM, respectively. [e] E710A/E883A double-mutant enzyme. [f] E710D/E883D double-mutant enzyme.

Determination of $K_{D(DNA)}$ in the presence and absence of the correct incoming dNTP allowed for a direct comparison of enzyme−TP binding as well as ternary complex formation between WT and mutant enzymes (Table 1). Also, this analysis provided a comparison between the conserved and nonconserved substitutions at each of the four carboxylate sites. Enzyme−TP binding was nearly unchanged in all mutant enzymes, except for E710A (and E710A/E883A), which exhibited a 3−4-fold reduction in TP binding affinity.

For the WT enzyme, the presence of the complementary dNTP (dGTP in this case) resulted in an ∼4-fold increase in TP binding affinity. A similar increase was noted for the E883A and E883D enzymes (Table 1). With the D882A and D882E substitutions, the magnitude of the response to the presence of complementary dNTP was ∼2-fold, while D705A and E710A showed no increase in their TP binding affinity under similar conditions (Table 1). E710D, however, exhibited a WT-like response to the complementary dNTP, suggesting that the carboxylate function at this position appears to participate in the prepolymerase events. Since noncomplementary nucleotides are known to destabilize

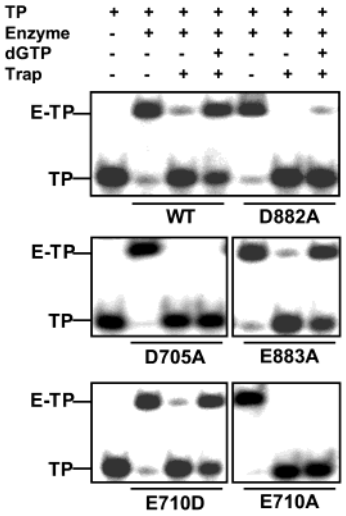

FIGURE 4: Formation of stable ternary complexes by carboxylate mutants. The ability of the carboxylate mutant enzymes (see Table 1) to form stable ternary complexes was assessed using a 5′-^{32}P-labeled, 3′-dideoxyribose-containing 21-mer primer annealed to its corresponding 33-mer template. Preformed enzyme−TP binary or enzyme−TP−dGTP ternary complexes were mixed with a 250-fold molar excess of nonradiolabeled TP (Trap) and electrophoresed under nondenaturing conditions as described in Experimental Procedures. For each mutant enzyme, a concentration sufficient to bind ~90% of the TP was used. Results obtained with D882E and E883D were similar to those obtained with D882A and E883A, respectively. Similarly, E710D/E883D and E710A/E883A mutant enzymes exhibited a pattern similar to those of E710D and E710A, respectively.

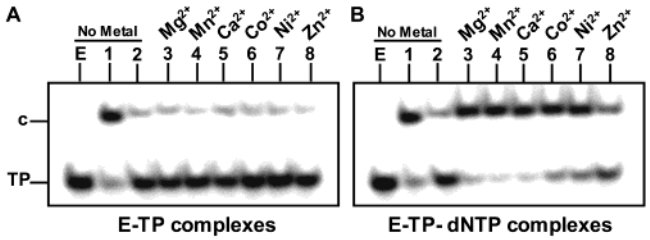

FIGURE 5: Noncatalytic divalent cations support ternary complex formation. Enzyme−TP (5′-^{32}P-labeled, 3′-dideoxyribose-containing 21-mer primer annealed to its corresponding 33-mer template) binary complexes and enzyme−TP−dGTP ternary complexes were formed in the presence of the indicated divalent cation. To preformed binary and ternary complexes NaCl was added to a final concentration of 0.5 M, and reaction mixtures were electrophoresed under nondenaturing conditions as described in Experimental Procedures. Panels A and B represent conditions with enzyme−TP binary and enzyme−TP−dGTP (100 μM) ternary complexes, respectively: lane E, control lane in the absence of enzyme; lane 1, complexes in the absence of metal ions; lane 2, complexes in the presence of 0.5 M NaCl; lane 3, complexes with 10 mM Mg^{2+} in the presence of 0.5 M NaCl; and lanes 4−8, complexes with 0.5 mM Mn^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}, respectively, in the presence of 0.5 M NaCl.

enzyme−TP complexes in the WT enzyme (*18*, *19*), we also examined the effect of mismatched dNTPs on the stability of enzyme−TP complexes with the E710A enzyme. While the WT showed an 8-fold increase in $K_{D(DNA)}$ in the presence of three mismatched dNTPs, E710A exhibited no change in $K_{D(DNA)}$ under similar conditions (Table 1, footnote d).

The sensitivity of binary and ternary complex formation to trap-TP competition in all nine mutant enzymes was also assessed. In this analysis, preformed enzyme−TP binary and enzyme−TP−dGTP ternary complexes were mixed with competing trap-TP, and the sensitivities of individual complexes formed with various mutant enzymes were compared to that of the WT. Enzyme−TP binary complexes with the WT, as well as all mutant enzymes, were fully sensitive to trap-TP competition. In the presence of dGTP, stable ternary complexes could not be formed with D705A, D882A, D882E, E710A, and E710A/E883A mutant enzymes, as judged by the significant loss of radiolabeled TP upon addition of trap-TP. On the other hand, ternary complexes formed with E883A, E883D, and E710D/E883D enzymes exhibited WT-like resistance to trap-TP (Figure 4). One of the striking differences in the properties of the D705A and D882A (as well as D882E) mutant enzymes is that D705A does not show an increase in $K_{D(DNA)}$ in the presence of dGTP (Table 1), and an enzyme−TP−dNTP complex formed with this enzyme is fully trap-sensitive (Figure 4). In contrast, D882A and D882E enzymes exhibit an ~2-fold increase in $K_{D(DNA)}$ in the presence of dGTP (Table 1), although the ternary complexes formed with these mutant enzymes exhibit near-complete sensitivity to trap-TP challenge (Figure 4).

Divalent Cation Requirement for Ternary Complex Formation. Polymerases are known to have an absolute requirement of divalent cations for substrate−dNTP binding as well as catalysis. In the case of *E. coli* DNA pol I, Mg^{2+} and Mn^{2+} are known to support the enzymatic activity of the enzyme, although a relaxation in the specificity of substrate selection with Mn^{2+} has been reported (*25*). When assessed for primer extension activity, divalent cations such as Ca^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} failed to support dNTP incorporation (data not shown). Since stable ternary complex formation in the presence of the correct dNTP was found to be Mg^{2+}-dependent (Figure 3), we examined the ability of these noncatalytic divalent cations to support ternary complex formation. For this assay, ternary complexes were distinguished from binary complexes on the basis of their sensitivity to 0.5 M NaCl (Figure 5). Preformed enzyme−DNA binary complexes with all divalent cations were found to dissociate with 0.5 M NaCl. In contrast, ternary complexes with the correct dNTP, formed in the presence of all the metal ions that were tested, were significantly salt-resistant. Ternary complexes with Zn^{2+} were relatively less resistant than those formed with other metals, as judged by the reduced amount of complex retained with Zn^{2+}.

DISCUSSION

In this communication, we have further clarified the roles of two essential aspartates (Asp^{705} and Asp^{882}) and their two neighboring glutamates (Glu^{710} and Glu^{883}) present in the active center of *E. coli* DNA polymerase I (Klenow fragment). We have previously shown that besides the two essential aspartates, the participation of a vicinal glutamate is required for optimal catalysis (*21*). Using conserved (Glu to Asp or vice versa) or nonconserved (Glu/Asp to Ala) substitutions of an individual carboxylate, we assessed the ability of various mutant enzymes to form enzyme−TP binary and enzyme−TP−dNTP ternary complexes. Our results reveal that, of the four carboxylates, only Glu^{710} participates in enzyme−TP binary complex formation, whereas Asp^{705}, Asp^{882}, and Glu^{710} are necessary for the formation of a stable ternary complex. The formation of a ternary complex also requires that the substrate dNTP be present in its metal chelate form.

Asp^{705} and Asp^{882} Are Required for Ternary Complex Formation. The inability of D705A and D882A (as well as D882E) enzymes to form a prepolymerase ternary complex is suggestive of the participation of both Asp^{705} and Asp^{882} in the stable binding of Mg^{2+}-chelated dNTP to an enzyme−TP binary complex. However, some differences in the properties of their mutant enzymes are noteworthy. Unlike the D705A enzyme, D882A and D882E mutant enzymes exhibit a 2-fold increase in the extent of enzyme−TP binding in the presence of the complementary dNTP (dGTP in this case) (Table 1). Also, a close examination of the trap-TP sensitivity of enzyme−TP−dNTP complexes formed with the Asp^{882} mutant enzymes consistently showed a minor (<5% of the original complex) fraction of the ternary complex that was resistant to TP challenge (in Figure 4, note the "shade of gray" in the trap-challenged ternary complex with D882A). Nevertheless, a majority of the preformed ternary complex is sensitive to the trap challenge, suggesting the requirement of Asp^{882} in the formation of a stable prepolymerase complex. These results are somewhat surprising, since one would expect that those mutant enzymes which exhibit an increase in TP binding affinity in the presence of substrate dNTP would form "WT-like", trap-resistant ternary complexes. The $K_{D(DNA)}$ and trap sensitivity differences between Asp^{705} and Asp^{882} mutant enzymes may represent different levels of involvement of the two aspartates in the formation of a stable ternary complex, with Asp^{705} playing a more direct and/or significant role than Asp^{882} in the prepolymerase events. Since Asp^{882} is a major contributor to the chemical step of bond formation (*26*), it appears likely that its participation in the formation of the prepolymerase ternary complex may also result in the closed conformation of the ternary complex. Some support to this interpretation may be found in the observation that the presence of the substrate dNTP increases the TP binding affinity of Asp^{882} mutant enzymes and that a minor fraction of their ternary complexes are trap-resistant. It is quite possible that during ternary complex formation, a conformational intermediate occurs between the open and closed forms, and that such an intermediate requires the participation of Asp^{882}.

The involvement of the two essential aspartates in the prepolymerase events is in good agreement with the available crystal structure data for pol I family polymerases. In the closed ternary complex structures of T7 DNA polymerase (*8*) and the large fragment of DNA pol I from *T. aquaticus* (Klentaq1) (*12*, *15*), both Asp^{705}- and Asp^{882}-equivalent aspartates are seen in contact with the substrate dNTP. These contacts are mediated via two independent metal ions, termed metal A and metal B. Metal A coordinates the Asp^{882}-equivalent aspartate with the α-phosphate of dNTP, as well as the 3′-OH of the primer terminus, while metal B binds to the Asp^{705}-equivalent aspartate and the $\beta-\gamma$ phosphate moieties of dNTP (*26*).

Glu^{710} Participates in the Formation of Binary and Ternary Complexes. The results obtained with E710A and E710D (Table 1) suggest that carboxylate function at this position aids in enzyme−TP binding, as judged by an increase in $K_{D(DNA)}$ for E710A, but an unchanged $K_{D(DNA)}$ for E710D compared to that of the WT enzyme. Using gel-shift analysis, Joyce and colleagues have shown that some of the major contributors to DNA binding affinity of the KF are His^{734}, Arg^{682}, and Tyr^{766}, as alanine substitutions of these residues result in 10-fold (H734A), 15-fold (R682A), and 22-fold (Y766A) increases in $K_{D(DNA)}$ values (*24*). Therefore, the 4-fold increase in $K_{D(DNA)}$ for the E710A enzyme (Table 1) appears to suggest an indirect interaction of this carboxylate with DNA, probably mediated via another side chain. For example, in the enzyme−DNA binary complex structure of Klentaq1 (PDB entry 4KTQ), the Glu^{615} side chain (Glu^{710} in the KF) is within 3.8 Å of Arg^{573} (Arg^{668} in the KF). The carboxylate side chain is likely to form a salt bridge with this invariant arginine, which forms two hydrogen bonds with the minor groove of DNA (*12*). It is also interesting to point out that a continuous 20 Å long hydrogen-bonding track involving Glu^{615}, together with Arg^{573}, Gln^{582}, Asn^{750}, Gln^{754}, and His^{784} (equivalent to Arg^{668}, Gln^{677}, Asn^{845}, Gln^{849}, and His^{881}, respectively, in the KF), can be seen in the enzyme−DNA binary complex structure of Klentaq1 (PDB entry 4KTQ). Furthermore, mutant enzymes with substitutions of these residues in the KF severely affect DNA binding (K. Singh et al., unpublished observations).

The carboxylate moiety of Glu^{710} appears to play a specific role in ternary complex formation. This is deduced from the behavior of E710A and E710D mutant enzymes in enzyme−TP−dNTP complexes. While E710D shows a ternary complex formation pattern similar to that of the WT enzyme (Table 1 and Figure 4), E710A shows no increase in the extent of TP binding in the presence of dGTP (Table 1), nor can this protein form trap-resistant ternary complexes (Figure 4). Since the conserved (Glu to Asp) substitution exhibits an unchanged phenotype with respect to ternary complex formation (Table 1 and Figure 4), the presence of a carboxylate group at position 710 appears to be more critical than the length of the side chain. The E710A mutant enzyme shows a rather intriguing phenotype with respect to incorrect dNTPs. In the case of the wild-type KF, the presence of noncomplementary dNTPs reduces enzyme−TP binding affinity, suggesting that the binding of a nonpairing nucleotide destabilizes enzyme−TP complexes (*18*, *19*). With the E710A enzyme, the presence of noncomplementary dNTPs did not destabilize preformed enzyme−TP binary complexes, as judged from the unchanged $K_{D(DNA)}$ in the presence of a dNTP mix containing dATP, dTTP, and dCTP (Table 1). Two inferences can be drawn from this result. The first inference is that in the absence of Glu^{710} (as in the E710A enzyme), dNTP binding is seriously affected, as a result of which correct or incorrect dNTPs have no effect on preformed enzyme−TP complexes. However, steady-state kinetic studies do not support this inference. The E710A substitution causes a moderate reduction in the substrate binding affinity as judged by a 3−5-fold increase in $K_{m(dNTP)}$ (*21*, *27*). The use of 100 μM dNTP in this study surely overrides the dNTP binding defect in E710A. Moreover, correct-dNTP-induced stabilization does not seem to be necessary for the catalytic activity of this enzyme, since a stable ternary complex is not formed with E710A (Table 1 and Figure 4). The second, and probably more pertinent, inference is that the inability of mismatched nucleotides to destabilize enzyme−TP complexes in E710A is likely to provide an opportunity for mismatch incorporation, unlike that in the WT enzyme. In fact, for the E710A enzyme, an increase in the error rate for nucleotide incorporation has been reported by Joyce and colleagues (*28*).

The role of Glu^{710} in the formation of a correct-dNTP-induced closed ternary complex can be explained using the available crystal structures of polymerases of the Pol I family. In enzyme−DNA binary complexes, an invariant tyrosine (the Tyr^{766} equivalent of the KF) occupies the position predicted for the first nucleotide of the 5′-template overhang [PDB entry 4KTQ (*12*); PDB entries 2BDP, 3BDP, and 4BDP (*29*)]. In the ternary complex structures of T7 DNA polymerase (*8*) and Klentaq1 (*12*, *15*), the Glu^{710}-equivalent residue (Glu^{480} in T7 and Glu^{615} in Klentaq1) has been seen to stabilize the phenolic ring of the tyrosine equivalent of Tyr^{766}. In the absence of this stabilization provided by Glu^{710} (as in E710A), the aromatic ring of Tyr^{766} will tend to occupy the position of the template nucleotide, as corroborated by its position in enzyme−DNA binary complexes. This would certainly hinder the proper positioning of substrate dNTP, thus preventing the formation of a stable ternary complex.

Glu^{883} Is Dispensable at the Prepolymerase Stage. The other neighboring glutamate, Glu^{883}, appears to have no functional role in the prepolymerase events, since enzymes with both conserved and nonconserved substitutions of this side chain exhibit a WT-like ability to form binary and ternary complexes (Table 1 and Figure 4). In the enzyme−DNA binary complex structures of Klentaq1 (*12*, *30*), the Glu^{883}-equivalent glutamate (Glu^{786}) does not make any direct or indirect contacts with the bound DNA. This observation is consistent with our results showing unchanged TP binding affinities in E883A and E883D enzymes.[2] In the ternary complex structures of T7 DNA polymerase (*8*) and Klentaq1 (*12*), the Glu^{883}-equivalent glutamate (Glu^{655} in T7 and Glu^{786} in Klentaq1) makes a water-mediated interaction with the phosphate backbone of the primer. Therefore, a reduction in the efficiency of ternary complex formation with the E883A enzyme was expected. Our results reveal that ternary complex formation in E883A and E883D remains unaffected (Figure 4). Hence, this interaction of Glu^{883} appears to be dispensable at the prepolymerase stage. Nonetheless, E883A and E883D substitutions are known to affect the catalytic efficiency of the enzyme (*21*, *27*), suggesting a role for Glu^{883} in the events following prepolymerase complex formation.

The assessment of ternary complex formation (Figure 4) is based upon dissociation or nondissociation of enzyme-bound radiolabeled TP, upon addition of excess TP, being a distinguishing feature between binary and ternary complexes. Obviously, the formation of a stable ternary complex requires the presence of both Mg^{2+} and substrate dNTP complementary to the template nucleotide. Romano and colleagues have demonstrated that noncomplementary dNTPs reduce the TP binding affinity of the enzyme as judged from $K_{D(DNA)}$ values (*19*). Therefore, the trap sensitivity of complexes with noncomplementary dNTPs (Figure 3) is probably due to the reversible nature of these complexes. In this context, two important observations are noteworthy. The first observation relates to the high stability of the ternary (but not enzyme−TP binary) complexes upon exposure to a high-ionic strength medium. As seen in Figure 2, radiolabeled TP in ternary complexes can withstand salt concentrations as high as 1 M, suggesting that TP stabilization in ternary complexes occurs via nonionic interactions. It is likely that the resistance to high salt in the ternary complex is due to an increased number of nonionic interactions with the template primer in the active site, or due to the restricted access to ionic interactions within the active site resulting from the conformational change accompanying ternary complex formation. Alternatively, stabilization of the single-stranded overhang by nonionic interactions in the ternary complex may result in salt resistance. Recently, we have identified Phe^{771} as the interacting site that binds the single-stranded overhang and stabilizes ternary complexes (A. Srivastava et al., unpublished results).

The second observation pertains to the ability of noncatalytic metal ions such as Ca^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} to support ternary complex formation. This finding suggests that any of these divalent cations can substitute Mg^{2+} in the prepolymerase complex, but catalysis requires the generation of a stereochemically appropriate transition-state species that can fit within the available space and the spatial charge distribution in the active site.

In summary, we have shown that both catalytically essential aspartates in *E. coli* DNA pol I are required for the formation of a stable prepolymerase ternary complex, and have discussed the differences in their involvement in complex formation. Of the two vicinal glutamates that form the carboxylate triads, only Glu^{710} is required for ternary complex formation, whereas Glu^{883} is dispensable at the prepolymerase stage.

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REFERENCES

1. Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., and Benkovic, S. J. (1987) *Biochemistry 26*, 8410−8417.
2. Englisch, V., Gauss, D., Freist, W., Englisch, S., Sternbach, H., and von der Haar, F. (1985) *Angew. Chem., Int. Ed. Engl. 24*, 1015−1025.
3. Joyce, C. M., and Steitz, T. A. (1994) *Annu. Rev. Biochem. 63*, 777−822.
4. Kuchta, R. D., Benkovic, P., and Benkovic, S. J. (1988) *Biochemistry 27*, 6716−6725.
5. Johnson, K. A. (1993) *Annu. Rev. Biochem. 62*, 685−713.
6. Eger, B. T., Kuchta, R. D., Carroll, S. S., Benkovic, P. A., Dahlberg, M. E., Joyce, C. M., and Benkovic, S. J. (1991) *Biochemistry 30*, 1441−1448.
7. Bryant, F. R., Johnson, K. A., and Benkovic, S. J. (1983) *Biochemistry 22*, 3537−3546.

[2] A 6-fold decrease in the DNA binding affinity of the E883A mutant enzyme compared to that of the WT enzyme has been previously reported by Joyce and colleagues (*27*). We have not been able to confirm this difference for the E883A mutant enzyme, either in a previous report (*21*) or in the current study. However, we would like to point out that $K_{D(DNA)}$ values differ widely depending upon the concentration and length of the TP used and the method employed for the binding assay. For example, Joyce and colleagues reported a $K_{D(DNA)}$ value of 8 nM for the WT KF using the DNase I protection assay with 0.1 nM M13 DNA annealed to a 25-mer primer (*27*). Using the same method, the same enzyme yielded a value of 0.2 nM when a self-annealing 68-mer was used (*24*). In our own experience, 3 nM self-annealing 37-mer yields a $K_{D(DNA)}$ value of 5 nM for the WT KF (*21*), while the use of 0.05−0.1 nM 33/21-mer substrate gives a $K_{D(DNA)}$ value of 0.5 nM (Table 1). The latter value is consistent with two other reports for the KF where low concentrations (0.015−0.1 nM) of TP have been used in the gel-shift assay (*18*, *24*).

8. Doublie, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998) *Nature 391*, 251−258.
9. Beese, L. S., and Steitz, T. A. (1991) *EMBO J. 10*, 25−33.
10. Beese, L. S., Friedman, J. M., and Steitz, T. A. (1993) *Biochemistry 32*, 14095−14101.
11. Beese, L. S., Derbyshire, V., and Steitz, T. A. (1993) *Science 260*, 352−355.
12. Li, Y., Korolev, S., and Waksman, G. (1998) *EMBO J. 17*, 7514−7525.
13. Li, Y., Kong, Y., Korolev, S., and Waksman, G. (1998) *Protein Sci. 7*, 1116−1123.
14. Li, Y., Mitaxov, V., and Waksman, G. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 9491−9496.
15. Li, Y., and Waksman, G. (2001) *Protein Sci. 10*, 1225−1233.
16. Patel, P. H., Suzuki, M., Adman, E., Shinkai, A., and Loeb, L. A. (2001) *J. Mol. Biol. 308*, 823−837.
17. Tong, W., Lu, C. D., Sharma, S. K., Matsuura, S., So, A. G., and Scott, W. A. (1997) *Biochemistry 36*, 5749−5757.
18. Dzantiev, L., and Romano, L. J. (1999) *J. Biol. Chem. 274*, 3279−3284.
19. Dzantiev, L., Alekseyev, Y. O., Morales, J. C., Kool, E. T., and Romano, L. J. (2001) *Biochemistry 40*, 3215−3221.
20. Arrigo, C. J., Singh, K., and Modak, M. J. (2002) *J. Biol. Chem. 277*, 1653−1661.
21. Gangurde, R., Kaushik, N., Singh, K., and Modak, M. J. (2000) *J. Biol. Chem. 275*, 19685−19692.
22. Derbyshire, V., Freemont, P. S., Sanderson, M. R., Beese, L., Friedman, J. M., Joyce, C. M., and Steitz, T. A. (1988) *Science 240*, 199−201.
23. Derbyshire, V., Grindley, N. D., and Joyce, C. M. (1991) *EMBO J. 10*, 17−24.
24. Astatke, M., Grindley, N. D., and Joyce, C. M. (1995) *J. Biol. Chem. 270*, 1945−1954.
25. Tabor, S., and Richardson, C. C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4076−4080.
26. Steitz, T. A. (1998) *Nature 391*, 231−232.
27. Polesky, A. H., Dahlberg, M. E., Benkovic, S. J., Grindley, N. D., and Joyce, C. M. (1992) *J. Biol. Chem. 267*, 8417−8428.
28. Minnick, D. T., Liu, L., Grindley, N. D., Kunkel, T. A., and Joyce, C. M. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 1194−1199.
29. Kiefer, J. R., Mao, C., Braman, J. C., and Beese, L. S. (1998) *Nature 391*, 304−307.
30. Eom, S. H., Wang, J., and Steitz, T. A. (1996) *Nature 382*, 278−281.

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