A Carboxylate Triad Is Essential for the Polymerase Activity of
Escherichia coli DNA Polymerase I (Klenow Fragment)

PRESENCE OF TWO FUNCTIONAL TRIADS AT THE CATALYTIC CENTER

Rajiv Gangurde, Neerja Kaushik, Kamalendra Singh, and Mukund J. Modak

The catalytic roles of two essential active-site asparagines at positions 705 and 882 of Escherichia coli DNA polymerase I have been well established (Steitz, T. A. (1998) Nature 391, 231–232). We now demonstrate that the participation of at least one additional carboxylate, a glutamate at position 710 or 883, is obligatory for catalysis. This conclusion has been drawn from our investigation of the properties of single (E710D, E710A, E883D, and E883A) and double (E710D/E883D and E710A/E883A) substitutions of residues Glu710 and Glu883. While single substitutions of either of the glutamates resulted in some reduction in polymerase activity, the mutant enzyme with simultaneous substitution of both glutamates with alanine exhibited a nearly complete loss of activity. Interestingly, substitution with two asparagines in place of the glutamates resulted in an enzyme species that catalyzed DNA synthesis in a strictly distributive mode. Pyrophosphorylolytic activity of the mutant enzymes reflected their polymerase activity profiles, with markedly reduced pyrophosphorylolytic activity by the double mutant enzymes. Moreover, an evaluation of Mg2+ and salt optima for all mutant enzymes of Glu710 and Glu883 revealed significant deviations from that for the wild type, implying a possible role of these glutamates in metal coordination as well as in maintaining the structural integrity of the active site.

The enzymatic process of DNA synthesis is a complex phenomenon. However, despite a large array of complexities and diversities, DNA polymerases from different sources and origins share some common mechanistic characteristics and appear to follow broadly similar rules for DNA synthesis. For example, mammalian DNA polymerase β, bacteriophage T7 DNA polymerase, human immunodeficiency virus reverse transcriptase, and Escherichia coli DNA polymerase I (pol I) employ a similar two-metal ion mechanism for polymerization (1, 2). In the proposed catalytic mechanism, one of the two metals (metal A) lowers the affinity of the primer 3'-OH for hydrogen, facilitating the 3'-O⁻ attack on the α-phosphate of the incom-
Klenow fragment (KF). Besides individual single mutations, conserved and nonconserved double mutations of Glu710 and Glu883 have been generated, and their properties have been investigated, in an attempt to pinpoint the effects of side chain alterations at these two sites. Results clearly indicate that in addition to the essential aspartates, the presence of at least one glutamate, at 710 or 883, is obligatory for DNA synthesis. Furthermore, the presence of this glutamate appears to be necessary for the processive mode of DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

Pfu Turbo polymerase for PCR amplification was purchased from Stratagene. PCR grade dNTPs, restriction endonucleases, and DNA-modifying enzymes were from Roche Molecular Biochemicals. Radiolabeled dNTPs were purchased from NEN Life Science Products. Biorex 70 cation-exchange resin was from Bio-Rad. The QiAquick PCR purification kit and QiAprep miniprep kit were from Qiagen. Synthetic oligomers used for PCR amplification, DNA sequencing, and activity assays (Sequences 1 and 2) were synthesized at the Molecular Biology Resource Facility at NJMS-UMDNJ (Newark, NJ) and were purified by preparative electrophoresis on a 12% (w/v) polyacrylamide-urea gel.

**Methods**

**In Vitro Mutagenesis**

A high level expression plasmid, pCJ141, which carries the KF insert with a D424A substitution to abolish the 3′, 5′ exonuclease activity (23), was used for site-directed mutagenesis. Synthetic oligomers, carrying the desired substitutions, were used as primers for PCR amplification of pCJ141 by Pfu Turbo polymerase, in accordance with the manufacturer’s protocol. The amplified product was purified using Qiagen’s PCR purification kit and treated with 10 units of Pfu Turbo polymerase, in accordance with the manufacturer’s protocol.

**Plasmids**

Plasmids containing mutations D705A, D705S, D882A, D882E, E710A, E710D, and E883A were a kind gift from Catherine Joyce (Yale University; see Ref. 11).

**In Vitro Mutagenesis**

In vitro mutagenesis was performed using the QuikChange site-directed mutagenesis kit and treated with 10 units of Pfu Turbo polymerase, in accordance with the manufacturer’s protocol. The amplified product was purified using Qiagen’s PCR purification kit and treated with 10 units of Pfu Turbo polymerase, in accordance with the manufacturer’s protocol.

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**Overproduction and Purification of WT and Mutant Enzymes**

Plasmid DNA from mutant clones was used to transfect E. coli CJ376, an expression strain used for this study (10, 26). Overexpression and purification of WT KF and its mutant derivatives was carried out with slight modifications of methods described previously (26, 27).

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**Enzyme Activity**

Template-directed DNA polymerization activity of WT and mutant enzymes was determined by two methods as follows.

**Trichloroacetic Acid Precipitation Assay**

The assay was performed with heteropolymeric 63/21-mer (Sequence 2) as well as homopolymeric (poly(dA)·(dT)18, (dC)63·(dG)18, or poly(A)·(dT)18) template-primer. A 100-μl reaction mixture contained 50 mM Tris-Cl (pH 7.8), 1 mM DTT, 40 μM respective substrate dNTP (mixed with 0.5 μCi/assay of α-32P-labeled dNTP or dGTP depending on the template-primer), 300 nM template-primer, and 7.5 mM WT/mutant enzyme. The reaction was initiated with MgCl2 (at 5 mM final concentration), allowed to proceed for 10 min at 37 °C, and quenched with 5% ice-cold trichloroacetic acid containing 10 mM Na-PPI. Trichloroacetic acid-precipitable DNA was collected on Whatman glass filters, washed with 70% ethanol, and dried, and radioactivity incorporated was determined by scintillation spectroscopy.

**Primer Extension Assay**

The ability of WT and mutant enzymes to extend a template-annexed primer was assessed on 63/21-mer heteropolymeric template-primer. The 21-mer primer was 5′-labeled using [γ-32P]ATP, gel-purified, and annealed to its corresponding 63-mer template in a 1:1 molar ratio. The assay mixture (9 μl) contained 50 mM Tris-Cl (pH 7.8), 1 mM DTT, 0.01% BSA, 5 mM NaCl, 50 μM concentration of each dNTP, and 150 nM annealed template-primer. The reaction was initiated with the addition of 180 nM WT/mutant enzyme, allowed to proceed at 25 °C for 30 and 60 s, and quenched by adding an equal volume of Sanger’s gel-loading dye (25). Five microliters of the quenched mix (approximately 6000 cpm/μl) was electrophoresed on a 16% (w/v) polyacrylamide-urea gel. Autoradiography of the gel and subsequent analysis were performed on a Molecular Dynamics PhosphorImager.

**Kinetic Parameters of WT and Mutant Enzymes**

Steady-state kinetic parameters, $k_{cat}$ and $K_m$ (dNTP), of the mutant enzymes were compared with those of WT, using conditions similar to the trichloroacetic acid precipitation assay. Heteropolymeric 63/21-mer template-primer (2 μM) was used, with 7.5 mM WT or 22.5–180 mM mutant enzyme. For each enzyme assay, a series of seven concentrations of a mixture of all four dNTPs was used, in order to bracket an expected $K_m$ range. Data analysis was essentially as described by Polesky et al. (10).

**Assay for Enzyme-DNA Binding**

Enzyme-DNA binding was examined by a gel shift assay, in which the ability of the parental electrophoretic migration of uncomplexed DNA was used to assess enzyme-DNA binding, with slight modifications of previously described methods (29, 30). 3′-32P-labeled self-anneling 37-mer DNA or a 21-mer heteropolymeric primer annealed to its corresponding 63-mer template was used at a final concentration of 3 nM (3′-OH termini). Enzyme-DNA mixtures, prepared in buffer containing 10 mM Tris-Cl (pH 8.2), 5 mM MgCl2, 0.05% (w/v) Nonidet P-40, and 10 mM glycerol, were incubated on ice for 15 min. For each enzyme, 8–10 enzyme concentrations were chosen in order to bracket the expected $K_d$ (DNA) range. The mixture was electrophoresed under nondenaturing conditions on a 6% (w/v) polyacrylamide gel prepared in 90 mM Tris borate buffer (pH 8.2) and prerun for 1 h at 120 V at 4 °C. Electrophoresis was carried out with 45 mM Tris borate buffer (pH 8.2) at 150 V for 4–5 h at 4 °C. Following electrophoresis, the gel was subjected to PhosphorImager analysis, and the distribution of radiolabeled DNA (free versus complexed) was assessed on a Molecular Dynamics PhosphorImager using ImageQuant.

**Determination of Salt and Magnesium Ion Optima for Polymerase Activity**

The optimal NaCl and Mg2+ ion concentrations required for DNA-directed DNA synthesis by the WT enzyme and its mutant derivatives were determined by the trichloroacetic acid precipitation assay. Homopolymeric poly(dA)·(dT)18 (annealed in a molar ratio of 1:2) was used at a final concentration of 300 nM.

For determination of salt optima, polymerase activity of WT and mutant enzymes was determined using NaCl concentrations in the range of 0–240 mM. For Mg2+ optima, MgCl2 concentrations ranged from 0.5 to 20 mM. For both studies, WT enzyme was used at a concen-
Fig. 1. Polymerase activity of carboxylate mutant enzymes. Effects of conserved and nonconserved mutations at Asp<sup>705</sup>, Asp<sup>882</sup>, Glu<sup>710</sup>, and Glu<sup>883</sup> were examined using a primer extension assay with 5<sup>9</sup>-<sup>32</sup>P-labeled 21-mer primer annealed to its corresponding 63-mer template. Lanes 1 and 2, products formed in 30- and 60-s reaction times, using 180 nM WT/mutant enzyme. Lane C, primer extension reaction without enzyme; lanes AA and DD, E710A/E883A and E710D/E883D double mutants, respectively.

63 mer

21 mer


c

WT

D705S

D882E

D882A

E710D

E883D

E710A

E883A

AA

DD

RESULTS

Of the four carboxylate residues at the polymerase active site of *E. coli* DNA polymerase I, two aspartates at positions 705 and 882 are known to be absolutely essential for catalysis (3). The other two carboxylates are glutamates at positions 710 and 883, which are the major focus of this investigation. We used site-directed mutagenesis to generate three types of mutant enzymes resulting from amino acid substitutions of these carboxylates. In the first type, an individual carboxylate was replaced by an alanine (D705A, D882A, E710A, or E883A); in the second type a single conserved substitution (D705S, D882E, E710D, or E883D) was effected; and the third type represented a double change, where two residues were simultaneously replaced with either an alanine or an aspartate (E710A/E883A or E710D/E883D). While the single mutants served to clarify the role of individual amino acids, the double mutants for Glu<sup>710</sup> and Glu<sup>883</sup> appeared to possess normal primer extension activity. Double mutations at these sites were not generated.

Since the focus of this study is on the polymerase active site, the WT enzyme and its mutant derivatives used in the investigation also contain the D424A substitution, which renders the WT/mutant enzyme exonuclease-deficient (23). Hence, WT represents an enzyme with the D424A mutation, while the E710A species contains both E710A and D424A substitutions.

All enzymes used in this study have been purified and quantified under identical conditions, and mutant derivatives described are similar to the WT in terms of yield, purity (~95% pure, as judged by Coomassie Blue staining of SDS-polyacrylamide gels) and solubility.

**Polymerase Activity of Carboxylate Mutants—**A primer extension assay was used as a qualitative measure to compare the DNA-directed DNA polymerase activity of mutant enzymes with that of the WT, using a heteropolymeric 63/21-mer template primer. Mutant enzymes containing substitutions at Asp<sup>705</sup> and Asp<sup>882</sup> failed to show any extension of the labeled primer (Fig. 1). On the other hand, single mutants of Glu<sup>710</sup> and Glu<sup>883</sup> appear to possess normal primer extension activity. For the WT enzyme, maximum accumulation of the 63-mer product was seen within 30 s of incubation, while the Glu<sup>710</sup> and Glu<sup>883</sup> mutants showed progressive accumulation of the full-length product requiring up to 60 s. Moreover, products shorter than 63-mer were clearly visible in all of the Glu<sup>710</sup> and Glu<sup>883</sup> lanes, implying an increased frequency of enzyme-DNA dissociation during catalysis. The E710D/E883D double mutant was more defective than individual single mutations, as judged by the accumulation of smaller length products (Fig. 1, lane DD). The E710A/E883A double mutant enzyme showed a complete loss of activity (Fig. 1, lane AA), similar to that seen with D705A and D882A mutants.

A standard acid precipitation assay was used to quantify the polymerase activity of mutant enzymes using homopolymeric...
Catalytic Carboxylates in DNA Polymerase I

DNA Binding Affinity of Glu<sup>710</sup> and Glu<sup>883</sup> Mutant Enzymes—In order to determine if the reduction in catalytic activity of mutant enzymes was related to their DNA binding affinity, we determined the dissociation constant (K<sub>D(DNA)</sub>) for WT and individual mutant enzymes using a gel shift assay (Fig. 3). A positional shift in the migration of DNA in the presence of increasing concentration of WT/mutant enzyme was monitored by nondenaturing polyacrylamide gel electrophoresis. A typical autoradiograph obtained for the WT enzyme depicts the migration of <sup>32</sup>P-labeled DNA at three positions (Fig. 3, center). Free uncomplexed DNA (marked U) was seen as a faster migrating band, while protein-DNA complexes were relatively slowly migrating (marked M). At higher enzyme concentrations (400 nM and above), enzyme-DNA dimers were formed, which migrated the slowest (marked D). A similar pattern was noted with all mutants of Glu<sup>710</sup> and Glu<sup>883</sup>. A plot of percentage of DNA complexed as a function of WT or mutant enzyme concentration was used for K<sub>D(DNA)</sub> determination (Fig. 3, bottom). For the WT enzyme, K<sub>D(DNA)</sub> was found to be in the range of 5–6 nM for both 37-mer self-annealing DNA (Table I) and the 63/21-mer template-primer (data not shown). E710D, E883A, and E883D mutant enzymes showed a moderate increase in K<sub>D(DNA)</sub>, whereas a 3–4-fold increase was shown by E710A and E710A/E883A mutant enzymes (Table I). Thus, it appears that the DNA binding affinity of the enzyme is not significantly altered by mutations at Glu<sup>710</sup> and Glu<sup>883</sup>.

Effects of Salt and Magnesium Ion Concentration on Catalyst—Salt and Mg<sup>2+</sup> are known to affect DNA and dNTP binding to the enzyme, thereby influencing the catalytic activity of DNA polymerases. Since carboxylate residues are likely to be involved in Mg<sup>2+</sup> binding and in the formation of salt-bridges with positively charged residues in their vicinity, we examined the effects of salt and Mg<sup>2+</sup> concentrations on the catalytic activity of these mutants.

Under the conditions of our assay, with hetero- as well as homopolymeric DNA, the WT enzyme exhibited optimal activity in the presence of 80–120 mM NaCl. Both Glu → Ala and Glu → Asp substitutions at Glu<sup>710</sup> and Glu<sup>883</sup> resulted in a significant decrease in the salt requirement of the enzyme, as judged by a steady decrease in polymerase activity with increasing NaCl concentrations (Fig. 4A). In the case of Mg<sup>2+</sup>, the optimal concentration required for WT activity was found to be 2.5 mM. Substitutions at Glu<sup>710</sup> and Glu<sup>883</sup> resulted in nearly

### Table I

| Enzyme | K<sub>m(DNTP)</sub> | k<sub>cat</sub> | K<sub>D(DNA)</sub> | Relative k<sub>cat</sub> | Relative K<sub>D(DNA)</sub> |
|--------|-----------------|--------------|----------------|---------------------|------------------|
| WT     | 3.9 ± 1.6       | 1.0          | 5.5 ± 0.4       | 1.0                 |                  |
| E710A  | 16.3 ± 0.7      | 4.2          | 17.2 ± 5.1      | 3.1                 |                  |
| E710D  | 5.9 ± 0.9       | 1.5          | 7.8 ± 1.0       | 1.4                 |                  |
| E883A  | 12.6 ± 1.7      | 3.2          | 7.1 ± 0.4       | 1.3                 |                  |
| E883D  | 10.8 ± 1.8      | 2.7          | 6.6 ± 0.4       | 1.2                 |                  |
| AA     | 43.6 ± 7.2      | 11.2         | 21.3 ± 5.7      | 3.8                 |                  |
| DD     | 9.6 ± 1.8       | 2.5          | 12.5 ± 1.9      | 2.2                 |                  |

<sup>a</sup> Determined by the trichloroacetic acid-precipitation assay as described under "Experimental Procedures." A heteropolymeric 63/21-mer template-primer was used at a final concentration of 2 μM. Enzyme concentrations were as follows: WT, 7.5 nM; Glu → Asp mutants, 22.5 nM; Glu → Ala mutants, 45 nM; E710D/E883D mutant (DD), 45 nM; E710A/E883A mutant (AA), 180 nM. Values are the average of two independent determinations.

<sup>b</sup> K<sub>m(DNTP)</sub> for mutant enzyme/K<sub>m(DNTP)</sub> for WT.

<sup>c</sup> k<sub>cat</sub> for WT/k<sub>cat</sub> for mutant enzyme.

<sup>d</sup> Determined by the gel shift assay (Fig. 3) using a 37-mer self-annealing DNA, as described under "Experimental Procedures." Values are the average of two independent determinations.

<sup>e</sup> K<sub>D(DNA)</sub> for mutant enzyme/K<sub>D(DNA)</sub> for WT.
5-fold increments in the optimal concentration of Mg$^{2+}$ required by individual enzymes (Fig. 4B). These results indicate that both Glu$^{710}$ and Glu$^{883}$ may participate in stabilizing intramolecular salt bridges and influence Mg$^{2+}$ coordination during some stage of the polymerization reaction.

**Mode of DNA Synthesis by Glu$^{710}$ and Glu$^{883}$ Mutant Enzymes**—Since all of the single and double mutants of Glu$^{710}$ and Glu$^{883}$ showed no significant difference in kinetic parameters, except for the alanine double mutant E710A/E883A, we investigated the mode of DNA synthesis by individual mutant enzymes on homopolymeric poly(dA)-(dT)$_{18}$ (Fig. 5) and heteropolymeric 63/21 template-primers (data not shown). A template challenge assay was used to assess the pattern of primer extension by mutant enzymes under conditions (4000-fold molar excess of DNA with heparin) restricting enzyme-DNA reassociation. The E710A/E883A mutant, which had no detectable primer extension activity, was not included in this study. For each assay, the concentration of the mutant enzyme was adjusted so that comparable catalytic activity was obtained in the absence of the trap (Fig. 5, lane U). The effectiveness of the trap was ascertained by complete inhibition of primer extension when enzymes were preincubated with the trap (Fig. 5, lane T).

On both poly(dA)-(dT)$_{18}$ and 63/21-mer template-primers, WT KF showed low processivity, amounting to the incorporation of approximately seven or eight nucleotides per enzyme-DNA encounter, which is in concurrence with processivity values reported earlier (10). E710D and E883D mutants showed no detectable processivity defect, as compared with the WT enzyme. However, removal of the acidic side chain, as in E710A or E883A mutants, was found to have compromising effects on the processivity of DNA synthesis (Fig. 5). Interestingly, the conserved double mutant, E710D/E883D, exhibited a strictly distributive pattern of synthesis, as judged by the extension of the labeled primer by merely one nucleotide (Fig. 5).

**Pyrophosphorolytic Activity of Carboxylate Mutants**—Since loss of carboxylate function at position 705 or 882 results in a complete loss of activity and substitutions at 710 or 883 alter the processivity of DNA synthesis, we examined pyrophosphorolytic activity of the carboxylate mutants to determine if these losses correlate with pyrophosphorolysis. Conceivably, loss of pyrophosphorolytic activity may indicate the inability of an enzyme to remove the pyrophosphate generated during phosphodiester bond formation, which may result in limiting the processivity of DNA synthesis.

Pyrophosphorolytic activity of the carboxylate mutants was assessed using homopolymeric poly(dA)-(dT)$_{18}$. Here, hydrolysis of the primer termini by individual mutant enzymes was monitored in the presence of sodium pyrophosphate. All mu-
were shown to affect overall catalysis only to a small extent (11), implying that these glutamates were not necessary components of the catalytic apparatus.

In this investigation, we first examined the catalytic activity of all four carboxylate mutant enzymes (Fig. 1) and then investigated the properties of the mutant enzymes of the two auxiliary glutamates by a variety of biochemical parameters, such as binding of DNA and dNTP, translocation, and processivity of DNA synthesis.

Results obtained from our activity assays and processivity studies are indicative of two requirements that are fulfilled by the auxiliary glutamates in the catalysis of DNA synthesis by the KF. The requirement for the participation of at least one carboxylate, in addition to the two essential aspartates (705 and 882) is evident from a nearly complete loss of activity noted with the E710A/E883A double mutant (Figs. 1 and 2). Glu → Ala single mutants of Glu710 and Glu883 retain 20–30% activity, which suggests that a two-aspartate/one-glutamate triad is functionally active, although with a reduced catalytic competence. Examination of kinetic constants for these single-mutant enzymes with 63/21-mer DNA suggests that single mutations have minor alterations in $K_M$ (dNTP). These results are in good agreement with previously reported $K_M$ (dNTP) values (11). The maximum change in $K_M$ (dNTP) was noted for E710A/E883A enzyme (Table I), which also exhibited the most pronounced defect in catalytic activity (Fig. 2). The pattern for $k_{cat}$ values (Table I) is consistent with the overall activity pattern seen with various substitutions at Glu710 and Glu883. Determination of $K_D$ (DNA) did not show significant differences between WT and mutant enzymes (Table I). Therefore, the participation of both Glu710 and Glu883 does not appear to be at the level of interaction with substrates.

It is reasonable to expect that carboxylic residues, with their negatively charged side chains, would make good candidates for divalent cation binding, and/or to make salt bridges with positively charged residues present in the immediate vicinity. Both types of reactions may be involved in maintaining the proper geometry of the active-site structure. The determination of salt optima for all Glu710 and Glu883 mutant species showed significant sensitivity to increasing salt concentration (Fig. 4), strongly suggesting that both Glu710 and Glu883 may be involved in salt bridge formation in the native enzyme. An examination of the available crystal structures of the pol I family of polymerases (31–34) has suggested that Glu710 may acquire the requirement by E710A and E710D mutants, suggesting a possible role of Glu710 in the initial binding of dNTP via Mg$^{2+}$ ions. In the case of Glu883, this residue appears to be well suited as a binding site for a divalent cation-mediated contact with the terminal phosphate of the primer strand. The fact that Mg$^{2+}$ optima is 40% activity as compared with WT. Homologous as well as heterologous double mutations at Glu710 and Glu883 exhibited a severe loss of pyrophosphorolytic activity (Fig. 6).

In contrast to the progressive decrease in catalytic activity with increasing salt concentration, all mutant species, including Glu → Asp substitutions, exhibited a 5-fold increase in the requirement of Mg$^{2+}$ to achieve optimal activity. Earlier, Joyce and colleagues (20) reported a similar increase in Mg$^{2+}$ requirement by E710A and E710D mutants, suggesting a possible role of Glu710 in the initial binding of dNTP via Mg$^{2+}$ ions. In the case of Glu883, this residue appears to be well suited to make a divalent cation-mediated contact with the terminal phosphate of the primer strand. The fact that Mg$^{2+}$ optima is shifted even with homologous (Glu → Asp) substitutions of Glu710 as well as Glu883 suggests that the shorter length of the aspartate side chain (as compared with glutamate) may not permit the formation of proper geometry of some intermediate involving carboxylate-bound Mg$^{2+}$.

Some insight into the possible role of Glu710 and Glu883 in the catalytic reaction is provided by differential processivity of DNA synthesis displayed by various mutant derivatives of these residues. Thus, another role for a vicinal carboxylate appears to be in the processive mode of DNA synthesis. We find

DISCUSSION

We have used site-directed mutagenesis to study the effect of side chain substitutions of four carboxylates in the active center of E. coli DNA pol I on the polymerase activity of the enzyme. For this purpose, 10 mutant enzymes, generated from conserved (glutamate to aspartate or vice versa) and nonconserved (glutamate to alanine) single and double mutations of the carboxylates in the KF, were examined for effects on catalytic activity and other properties. The foremost finding of this investigation is the elucidation that the participation of either Glu710 or Glu883 is essential for DNA synthesis by the KF.

An earlier study on the active-site residues of KF has demonstrated the essential requirement of two aspartates, Asp705 and Asp882, in DNA synthesis (11). This was deduced from a severe reduction in polymerase activity when either Asp705 or Asp882 was replaced by glutamate or alanine. The side chain substitutions of four carboxylates in the active center of E. coli DNA pol I were shown to affect overall catalysis only to a small extent (11), implying that these glutamates were not necessary components of the catalytic apparatus.

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that processive synthesis of DNA also requires the presence of one more carboxylate besides the two-aspartate/one-glutamate triad. Absence of the fourth carboxylate increases the frequency of enzyme-DNA dissociation during catalysis, as is evident from the decreased processivity observed with Glu → Ala single-mutant enzymes. WT-like processivity of the Glu → Asp mutants, 54 nM; Glu → Asp mutants, 108 nM; E710D/E883D double mutant, respectively.

Results obtained from an assessment of the pyrophosphorolytic activity of the mutant enzymes may be useful to detect additional defects in the catalytic pathway. Pyrophosphorolytic activities of the single mutants of 705, 882, 710, and 883 are generally representative of their polymerase activities. For example, the polymerase-deficient D705A and D882A mutant enzymes are unable to generate dNTP from the primer terminus in the presence of an excess of pyrophosphate, while E710A, which shows some reduction in polymerase activity, exhibits reduced pyrophosphorolytic activity (Fig. 6). However, the pyrophosphorolytic profile of the E710D/E883D double mutant is not a representation of its polymerase activity, which is about 30% that of the WT enzyme. Primer degradation by this mutant did not proceed beyond one nucleotide, which seems to correlate well with its mode of DNA synthesis. The inability of the enzyme to catalyze pyrophosphorolysis beyond the first nucleotide probably reflects its inefficiency to undergo the second conformational change essential for activity (35). During DNA synthesis, following the formation of a phosphodiester bond between the 3'-OH of the primer terminus and the 5'-PO₄ of the incoming nucleotide, the enzyme undergoes a conformational change (generally referred to as the second conformational change, in order to distinguish it from the first nonchemical change that precedes phosphodiester bond formation) and translocates along the DNA to expose the next template-base (35, 36). A severe defect in the pyrophosphorolytic activity, which is essentially a reversal of the forward reaction, is indicative of an inability of the enzyme to undergo the second conformational change. Mutant enzymes defective in undergoing this conformational change may also be expected to be translocation-deficient, which in turn may lead to the destabilization of the enzyme-DNA complex. Hence, mutant enzymes with this defect would dissociate from DNA at a frequency higher than the WT enzyme, leading to a distributive mode of DNA synthesis.

In summary, the presence of at least three carboxylates in the active site of E. coli DNA pol I seems to be obligatory for optimal DNA synthesis. Furthermore, processive DNA synthesis appears to require the presence of at least one carboxylate in KF. The demonstration of the requirement of a carboxylate triad for catalysis in pol I type enzymes also unifies this class with the reverse transcriptase class of polymerases, where the requirement of three carboxylates is well established (13, 37). DNA pol I appears to possess two active carboxylate triads: Asp⁷⁰⁵-Glu⁷¹⁰-Asp⁸⁸² and Asp⁷⁰⁵-Asp⁸⁸²-Glu⁸⁸³. At this point,
however, specific conditions dictating the selective utilization of one triad or the other remain unclear.

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