Side Chains Involved in Catalysis of the Polymerase Reaction of DNA Polymerase I from *Escherichia coli*

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Andrea H. Polesky‡, Michael E. Dahlberg‡, Stephen J. Benkovic‡, Nigel D. F. Grindley, and Catherine M. Joyce‡

From the Department of Molecular Biophysics and Biochemistry, Yale University Medical School, New Haven, Connecticut 06510 and the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

To continue our mutagenesis study of the polymerase active site of the Klenow fragment of DNA polymerase I, we have characterized new mutants with substitutions at Asp<sub>705</sub>, Glu<sub>710</sub>, and Glu<sup>833</sup>, and have investigated further the mutations in residues Arg<sub>688</sub>, Gln<sub>840</sub> and Asp<sub>862</sub>, which showed strong k<sub>cat</sub> effects in our previous study. To determine which step of the reaction is rate-limiting for each mutant protein, we measured the effect on the reaction rate of an α-thio-substituted dNTP. One group of mutants showed a substantial sulfur elemental effect, while a second group, like wild-type Klenow fragment, was unaffected by the phosphorothioate substitution. Consistent with earlier data, these results imply that, between formation of the enzyme-DNA-dNTP ternary complex and completion of phosphodiester bond formation, there are two kinetically distinct steps, only one of which is sensitive to sulfur substitution. The rather complex elemental effect data obtained with the mutant derivatives of Klenow fragment are more consistent with the elemental effect being the result of a steric clash in one of the transition states than with explanations based on electron negativity differences between sulfur and oxygen. The data suggest that the side chain of Asp<sub>862</sub> is involved in the proposed steric clash, and that Gln<sub>840</sub> and Gln<sub>833</sub> participate in the sulfur-sensitive step of the reaction. Based on our results, and comparisons with other phosphoryl transfer enzymes, possible mechanisms for the polymerase reaction are discussed.

The Klenow fragment of DNA polymerase I from *Escherichia coli* provides a simple and well-characterized model system for studying template-directed DNA synthesis and identifying which amino acid side chains participate in substrate binding and catalysis of phosphodiester bond formation. Klenow fragment is particularly appropriate for structural studies because it is a single-subunit enzyme and the only polymerase of known three-dimensional structure (Kornberg, 1980; Ollis et al., 1985). The kinetic pathway of the polymerase reaction catalyzed by Klenow fragment has been extensively studied and rate constants have been determined for many of the individual steps of the reaction (McClen and Jovin, 1975; Bryant et al., 1983; Mizrahi et al., 1985; Kuchta et al., 1987, 1988; Carroll and Benkovic, 1990; Dahlberg and Benkovic, 1991). Furthermore, a recent protein sequence comparison has highlighted three conserved sequence motifs in both bacterial and eukaryotic DNA polymerases, suggesting that the important active site residues may be common to the family of DNA-dependent DNA polymerases (Delarue et al., 1990).

Crystallographic and biochemical studies have demonstrated that the two activities of Klenow fragment, the 5′,3′-DNA polymerase and the 3′,5′-exonuclease, reside on distinct structural domains (Ollis et al., 1985; Freemont et al., 1986; Derbyshire et al., 1988; Polesky et al., 1990). The larger C-terminal domain, which carries the polymerase activity (Freemont et al., 1986), also contains a large cleft. Based on structural, electrostatic, and genetic arguments, this cleft has been proposed as the binding site for the duplex DNA substrate of the polymerase reaction (Joyce et al., 1985; Ollis et al., 1985; Warwicker et al., 1988). In the absence of direct crystallographic data on the binding of substrates, products, or inhibitors to the polymerase domain, site-directed mutagenesis has been valuable in locating the polymerase active site within the region deduced from biochemical studies (Joyce and Steitz, 1987; Polesky et al., 1990). Determination of the steady-state parameters K<sub>cat(DNTPT)</sub>, k<sub>cat</sub>, and K<sub>DNTPT</sub> for the mutant Klenow fragment derivatives produced in this initial study demonstrated that they can be classified in two different groups (Fig. 1). Mutations at residues Tyr<sup>266</sup>, Arg<sup>641</sup>, and Asn<sup>665</sup> cause an increase in K<sub>cat(DNTPT)</sub>, implying that, in this system, is a good measure of K<sub>DNTPT</sub> (Eger et al., 1991), suggesting that these residues may interact with some part of the incoming dNTP molecule. Mutations in Arg<sup>665</sup>, Gln<sup>849</sup>, and Asp<sup>862</sup> cause a large decrease in k<sub>cat</sub> (100- to 1000-fold), implying that these side chains play a role in the polymerase reaction after substrate binding has taken place. Most of these mutations, which are in residues forming the surface of the proposed DNA-binding cleft, have an effect on K<sub>DNTPT</sub>, implying that there are multiple close contacts between protein and DNA.

To continue our study of residues that are directly involved in catalysis of the polymerase reaction, the present study poses two questions. First, are there additional side chains,
within the fairly small region defined by Asp⁶⁶, Glu⁶⁷, and Asp⁹⁰, that participate in catalysis? To address this question, we made a new group of mutations in the neighboring residues, Asp⁷⁰⁵, Glu⁷¹⁰, and Glu⁸⁰³, all of which are close to the turn connecting β-strands 12 and 13 in the tertiary structure (Fig. 1). Biochemical analysis of the resulting mutant proteins indicated that mutations in these additional residues all cause a decrease in the steady-state k₄₆ value.

Our second question was to determine which of the residues implicated by the k₄₆ determinations are directly involved in phosphodiester bond formation. Direct comparison of mutant and wild-type k₄₆ values is not necessarily informative since the mutations may cause a change in the rate-limiting step. As illustrated in Fig. 2, the polymerase reaction pathway is quite complex. After the ordered addition of DNA followed by dNTP, the ternary complex undergoes a conformational change (k₅) before phosphodiester bond formation (k₆); a subsequent conformational change (k₇) precedes the product release steps. Clearly, a mutation that reduces the steady-state k₄₆ does not necessarily influence the rate of the chemical step that precedes or follows these other steps. In order to identify active site residues which play a direct role in phosphodiester bond formation, our goal was to determine the effects of the mutations under study on the chemical step of the reaction, using single turnover conditions. To probe the relative rates of individual steps of the polymerase reaction, we compared the rate of addition of a deoxyribonucleoside 5′-O-(1-thiotriphosphate) analog (dNTpS) with that of the normal dNTP substrate. In the past, such experiments have been interpreted fairly simply, based on the assumption that the lower electronegativity of sulfur compared with oxygen would slow the reaction of the phosphorothioate analog in an associative nucleophilic substitution of the type proposed for the polymerase reaction (Mizrahi et al., 1985). However, a slower reaction of the phosphorothioate analog would indicate that phosphodiester bond formation is at least partially rate-limiting. More recent model studies (Herschlag et al., 1991) suggest that this assumption may not be applicable to reactions of this type, and thus one cannot assume a priori that the sulfur elemental effect is a probe for the chemical step of the reaction. Fortunately, a recent kinetic study of wild-type Klenow fragment (Dahlgren and Benkovic, 1991) provides independent evidence, allowing us to infer that, in this system, the elemental effect is indeed diagnostic for the chemical step of the reaction. Thus, the earlier conclusion of Mizrahi et al. (1985) remains valid. Based on the lack of a sulfur elemental effect in the forward reaction, these authors had inferred that for wild-type Klenow fragment a nonchemical step, assigned to the conformational change (k₅), is slower than phosphodiester bond formation. The data reported below identify mutations that affect the chemical step of the polymerase reaction and indicate that the sulfur elemental effect may have a substantial steric component. These conclusions allow us to speculate about the layout of the active site and the mechanism of the polymerase reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Poly(dA), average length 290 residues, oligo(dT)₁₀, ultrapure dNTPs, ultrapure dideoxyNTPs, dGTPαS, and dATPαS (both a mixture of Sp and Rp diastereomers) were purchased from Pharmacia LKB Biotechnology Inc. The pure Sp diastereomers of dATPαS and dTTPαS were prepared as described previously (Polesky et al., 1990). The dideoxy products were visualized by Western blotting (Sambrook et al., 1989). The pre-steady-state kinetics of the polymerase reaction and measurement of the effect of thiophosphoryl substitution—Two reactions were studied. One was the addition of a T residue to oligo(dT)₁₀ previously phosphorylated using T₄ polynucleotide kinase, DNA ligase, and restriction endonucleases were from New England Biloars or Boehringer Mannheim and were used according to the accompanying instructions.

**Thermolysin Digestion of Mutant Derivatives of Klenow Fragment**—The sensitivity of each mutant protein to thermolysin digestion at a series of temperatures (Hecht et al., 1983) was compared to that of wild-type Klenow fragment as previously described (Polesky et al., 1990). The digestion products were visualized by Western blotting (Sambrook et al., 1989).

**Pre-steady-state Kinetics of the Polymerase Reaction and Measurement of the Effect of Thiophosphoryl Substitution**—Two reactions were studied. One was the addition of a T residue to oligo(dT)₁₀ previously phosphorylated using T₄ polynucleotide kinase, and the other was the addition of a Met residue to dATPαS. The 17-mer universal sequencing primer was annealed to single-stranded DNA of a phage M13 mp19 derivative. In either case, the primer was 5′-phosphorylated using T₄ polynucleotide kinase and [γ-³²P]ATP, and...
then annealed to a 3-5-fold excess of the template DNA in a buffer containing 10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, for 15 min at 55 °C followed by 1 h at room temperature.

A typical reaction contained 1–4 nM primer, 52.5 μM dNTP, or 105 μM dNTPαS (a mixture of the S and Rp diastereomers), 10 mM dithiothreitol, 2 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, and 5% (v/v) glycerol. When using poly(dA)-oligo(dT)₆, each reaction mixture also contained 5′ end-labeled oligo(dC)₁₈ (approximately 5 nM) to serve as an internal standard for quantitation. The reaction was initiated by addition of enzyme to a final concentration of at least 0.67 nM. All reactions were carried out at 22 °C. Samples (4 μl) were removed at appropriate time intervals, quenched with 6 μl of stop solution (67% (v/v) deionized formamide, 0.007% (w/v) of bromphenol blue and xylene cyanol FF, 0.33% (w/v) sodium dodecyl sulfate, and 40 mM EDTA), and then frozen in dry ice. The samples were boiled for 1 min, cooled in ice-water, and fractionated by electrophoresis on 17.5% polyacrylamide-urea sequencing gels. The gels were dried down and exposed to Kodak X-AR film.

The total amount of starting material (DNA₀) remaining in each reaction as a function of time was determined by densitometric scanning and integration using an LKB Ultrascan laser densitometer. In the universal primer reactions, the amount of starting material was measured as a percentage of the total DNA bands in each lane, thus compensating for lane to lane variability in the amount of sample loaded. In the homopolymer reactions, the amount of oligo(dT)₆ was measured relative to the internal control, oligo(dC)₁₈. The small amount (≤5%) which remained unannexed in each batch of substrate was subtracted from each measurement. The rate of disappearance of the starting material (the rate of DNA₀→DNA₀ₚ) in the presence of dNTP or dNTPαS was determined by plotting ln[(%DNA₀ remaining) versus time. Since the rate could be fit to a single exponential, the resulting plot was linear, with the slope (determined by a least-squares analysis) corresponding to the first-order rate constant, kₐᵦ (see Table II). The rate of addition of the first two nucleotides (DNA₀→DNA₁ₚ) was determined by plotting the quantity ln[(%DNA₀ + DNA₀ₚ remaining) versus time. Each experiment was done at least twice.

**Quenched-flow Kinetic Analysis of the Polymerase Reaction**—Additional pre-steady-state kinetic analysis of the polymerase reaction was carried out for the E710A, H881A, Q849A, and E883A mutant enzymes, using quenched-flow techniques. The DNA substrate used in these experiments was a synthetic oligonucleotide duplex, the 13/20-mer, whose preparation, purification, and quantitation have been described previously (Kuchta et al., 1987; Dahlberg and Benkovic, 1991). Klenow fragment and 5′ end-labeled 13/20-mer were preincubated at a molar ratio of 1:5 (enzyme:DNA) in a total volume of 30 μl of 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA (typical DNA concentrations ranged from 200 to 800 nM). The reaction was initiated by mixing with an equal volume of dATP or dATPαS (50 mM dNTPαS (50% diastereomer) in 50 mM Tris-HCl, pH 7.5, 12.5 mM MgCl₂) in a rapid-quenchant apparatus, the design of which has been described previously (Johnson, 1986). The final dATP or dATPαS concentrations after mixing were 10, 30, and 50 μM. The reaction was quenched with 0.1 M EDTA at time points between 0 and 5 s and the quenched samples were fractionated by gel electrophoresis. Areas of the gel corresponding to 13-mer and 14-mer for each time point were excised and counted. The resulting time course was analyzed by computer simulation of the data using the program KINSIM (Barshoff et al., 1983) as modified by Anderson et al. (1988).

**RESULTS**

**Purification and Characterization of New Mutant Proteins**—Fig. 1 indicates the positions on the Klenow fragment structure of the amino acid residues that have been mutated in this and our previous study. The positions of the new mutations made for the present study (Asp₇⁰⁵, Glu₇¹⁰, and Glu₈⁸³, shown on a shaded background in Fig. 1) were chosen by examining the region of the Klenow fragment structure near those residues (Arg₈⁹₈, Glu₄⁸⁴, and Asp₈⁸₂, where previous mutations resulted in a decrease in kₐᵦ (Polesky et al., 1990). Two types of amino acid substitutions were made. In the first type, each amino acid was changed to alanine (D705A, E710A, and E883A) thus removing the functional group. The second type of mutation was more conservative, restoring all or part of the original functional group (D705S and E710D) to see if any activity lost as a result of the alanine substitution could be regained. The new mutations were made by oligonucleotide-directed mutagenesis and were then subcloned into a plasmid vector for protein overproduction, as previously described (Polesky et al., 1990). Based on the experience in our earlier study, all the mutations were studied in combination with the D424A mutation which abolishes 3',5'-exonuclease activity (Derbyshire et al., 1988) and therefore eliminates complications due to this competing reaction. The mutant proteins were purified by a modification of the procedure described by Derbyshire et al. (1991) (see "Experimental Procedures").

The steady-state kinetic parameters kₐᵦ and Kₐᵦ₃ₕNTP were measured in the presence of a saturating concentration of poly(dA)₉₋₁₀-DNA (dATP) for each mutant protein. The DNA dissociation constant, Kₐᵦ₃ₕDNA, was measured by DNase I footprinting on a 3′ end-labeled oligonucleotide annealed to M13 as described previously (Polesky et al., 1990). Table I contains the data for the new mutant proteins, together with a summary of the kinetic parameters for wild-type Klenow fragment and the mutant derivatives from our previous study for comparison (Polesky et al., 1990). The new fast protein liquid chromatography purification method decreased the assay background from the level in our previous study, allowing us to measure Kₐᵦ₃ₕNTP and kₐᵦ for the D882S mutant protein, whose activity was previously at the assay background. These values are also reported in Table I. The new mutations all caused a decrease in the affinity of the enzymes for DNA of between 5- and 30-fold compared with wild-type Klenow fragment. Mutations at Asp₇⁰⁵ and Glu₇¹⁰ increased Kₐᵦ₃ₕNTP, which suggests that these residues are involved in dNTP binding. Mutations at Asp₇⁰⁵ also caused a 1000-fold decrease in kₐᵦ implying that this amino acid may play an important role in catalysis.

In order to assess the structural integrity of the new mutant Klenow fragment derivatives, the sensitivity of each protein to thermolysin digestion was examined at a series of temperatures between 22 and 70 °C, as described previously (Polesky et al., 1990). No changes were observed from the wild-type pattern when the digestion products were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Additional circumstantial evidence that...
these mutations do not cause gross distortions of the protein structure is provided by the overproduction yields and the solubility and chromatographic properties of the mutant proteins, all of which were identical to wild type. Moreover, none of the mutations at Glu710 or Glu883 gave a temperature-sensitive phenotype in vivo. The phenotype in vivo of mutations at Asp882 could not be assessed since these alleles could not be isolated in a polA-deficient strain, just as we observed previously for mutations at Asp882 (Polesky et al., 1990). Several of the mutant proteins are likely to be candidates for crystallographic studies in the future, at which time a more detailed examination of their structures can be accomplished.

Further Kinetic Analysis of the Polymerase Reaction—Because of the complexity of the polymerase reaction (Fig. 2), the steady-state $k_{cat}$ is of very limited use in making quantitative comparisons between wild-type and mutant proteins, since the rate-limiting step may be different in the two cases. We therefore used the steady-state $k_{cat}$ values as a rough indicator of those mutant Klenow fragment derivatives that deserved further kinetic analysis. Mutant proteins having a substantial reduction in $k_{cat}$ (R668A, D705S, D705A, D882A, D882E, and D882S) were analyzed further to pinpoint the rate-limiting step. Since Glu710, His891, and Glu883 are close neighbors, in the tertiary structure, of the residues already implicated in catalysis, the E710A, H881A, and E883A mutant proteins were also included in these experiments. This mutation causes a large decrease in $k_{cat}$, although a more drastic mutation at the same position (N845A) gives a $k_{cat}$ that is very similar to that of wild-type Klenow fragment (Table I). We therefore reasoned that the N845D mutation is unlikely to exert a direct effect on the chemical step, and that the inclusion of this protein in our studies could be useful in alerting us to spurious effects unrelated to chemical catalysis.

The number of steps contributing to the observed reaction rate can be simplified by carrying out the polymerase reaction

| Protein | $K_{M,ONTP}$ | $K_{M,ONTP}$ | $k_{cat}$ | $K_{DNA}$ | Relative DNA affinity |
|---------|--------------|--------------|-----------|-----------|----------------------|
|         | Average | Range* | Average | Range* | Average | Range* | Average | Range* | Average | Range* |
| Wild-type | 2.8 | 2.4 | 8 | 1 | 0.06 |
| D705A | 10 | 8.2-13 (2) | 0.001 | 0.0009-0.0002 (2) | 160 | 0.05 |
| D705S | 8.9 | 6.2-12 (2) | 0.003 | 0.002-0.003 (3) | 45 | 0.2 |
| E710A | 15 | 12-18 (2) | 0.1 | 0.09-0.1 (2) | 250 | 0.03 |
| E710D | 7.7 | 6.7-8.7 (2) | 1.7 | 1.1-2.3 (3) | 110 | 0.07 |
| E883A | 4.8 | 3.6-6.0 (2) | 0.09 | 0.05-0.2 (3) | 50 | 0.2 |
| R668A | 6.5 | 0.006 | 150 | 0.05 |
| Y766S | 6.4 | 0.8 | 13 | 0.6 |
| R814A | 9.8 | 0.3 | 53 | 0.2 |
| N845A | 23 | 1.0 | 5 | 1.7 |
| N845D | 1.7 | 0.03* | 55 | 0.2 |
| Q849A | 3.8 | 0.02 | 160 | 0.05 |
| Q849E | ND | 0.001 | 91 | 0.09 |
| H881A | 3.3 | 0.3 | 28 | 0.3 |
| D882A | 4.1 | 0.006 | 0.5 | 16 |
| D882E | 2.1 | 0.007 | 6 | 3 |
| D882N | ND | <0.0001 | 30 | 0.6 |
| D882S | 7.5 | 6.2-8.7 (2) | 0.001 | 0.0001-0.002 (3) | 0.9 | 9 |
| D424A | 8,18 | | | |

* The number of determinations is given in parentheses.

* Data from two or three independent experiments were combined to calculate $K_{DNA}$. Differences of 2-fold or less in $K_D$ are not considered significant. Except where noted, the 3' end-labeled substrate, 5'-d-[ACCAGTTATCGGCTACCTGCATGA]-3', annealed to a complementary M13 template, was used. In this study, $K_D$ was determined by fitting the data as indicated under "Experimental Procedures." This has resulted in small changes in the previously reported $K_{DNA}$ values which were determined by interpolation (Polesky et al., 1990).

* The dissociation rate from DNA was measured by rapid-quench techniques for four mutant proteins, using the 13/20-mer substrate.

* The average $K_{M,ONTP}$ and $k_{cat}$ values from our previous study are reported without ranges.

* The values of $K_{M,ONTP}$ and $k_{cat}$ for wild-type Klenow fragment are the average of the two values previously reported (Polesky et al., 1990) for two different preparations of the wild-type protein.

* Reported by Kuchta et al. (1987) for the 13/20-mer substrate.

* The small change from our previously published value of $k_{cat}$ for the N845D mutant protein is due to the inclusion of new data.

* ND, not determined.

* The 5' end-labeled oligonucleotide, 5'-d-[ACCAGTTATCGGCTACCTGCATGA]-3', annealed to a complementary M13 template was used. In this study, DNA was not included in the reaction as indicated under "Experimental Procedures." This has resulted in small changes in the previously reported $K_{DNA}$ values which were determined by interpolation (Polesky et al., 1990).

* Activity was at, or close to, the assay background.

* In our previous study, the activity of the D882S mutant protein was at the background level of the assay. Subsequently, the purification of mutant proteins by FPLC methods has reduced the assay background to 0.0001 s$^{-1}$ enabling us to measure the activity of D882S.
under conditions of protein excess. In this situation, the observed rate for the addition of the first nucleotide, which can be monitored by following the elongation of a labeled primer on a polyacrylamide-urea gel, only reflects the steps up to and including phosphodiester bond formation, but not any of the steps following the chemical step (Fig. 2). Under these pseudo first-order conditions, the data are expected to fit a single exponential.

Our first question was whether the rate-limiting step for each mutant derivative precedes or follows the point of bond formation. To answer this question, the rate of addition of the first nucleotide (DNA,=DNA,+,) was compared to the rate of addition of the first two nucleotides (DNA,=DNA,+,). If the time taken to add two nucleotides is substantially longer than twice the time taken to add one nucleotide, so that the DNA,+, product accumulates before the DNA,+, product appears, it suggests that the rate-limiting step for the Klenow fragment derivative under consideration follows the chemical step of the reaction. To avoid sequence context effects which could differentially affect the rate of the reaction and the affinity of the enzyme for the DNA at each successive dNTP addition step, the homopolymer poly(dA)-oligo(dT) was used as the substrate. The first time course in Fig. 3, panel A, shows an example of the data obtained, which was analyzed by densitometry. The rate of addition of the first nucleotide (DNA,=DNA,+,), determined by measuring the rate of loss of the primer, was calculated by fitting the data to a single exponential. The rate of addition of the first two nucleotides (DNA,=DNA,+,) was similarly determined by measuring the rate of loss of the primer and the DNA,+, product. For the R668A, D705A, D705S, D882A, D882E, and D882S proteins, the time taken to add two nucleotides was approximately twice the time taken to add one nucleotide, suggesting that the rate-limiting step occurs before or at the point of phosphodiester bond formation (data not shown). For the other mutant proteins under investigation (E710A, N845D, Q849A, H881A, and E883A), the reaction was too fast to allow an accurate comparison of the rates of addition of the first two nucleotides using our current experimental protocol, since the DNA,+, product could be detected only transiently. Quenched-flow studies on the E710A, Q849A, H881A, and E883A mutant proteins, albeit using a different DNA substrate, implied that, for these proteins, the rate-limiting step may follow the chemical step (see below).

Thus, for the mutant proteins having changes at Arg<sup>668</sup>, Asp<sup>705</sup>, and Asp<sup>882</sup>, the rate-limiting step of the polymerase reaction must be substrate binding (k<sub>1</sub> or k<sub>2</sub>, Fig. 2), the conformational change (k<sub>3</sub>), or bond formation itself (k<sub>4</sub>). It is unlikely that either of the substrate binding steps is rate-limiting since the rates of DNA and dNTP binding by wild-type Klenow fragment are in the range of diffusion-limited rates (Kuchta et al., 1987; Eger et al., 1991). Although several of the mutant proteins under discussion have higher K<sub>DDNA</sub> values than the wild-type protein, the change in DNA affinity is 30-fold at the most (Table I). Even if the change in DNA affinity were completely reflected in the association rate, which is unlikely, this step would remain kinetically invisible. This conclusion is supported by the control experiment (de-
scribed below), carried out on some of the mutant proteins, which showed that the reaction rate was not affected by a change in DNA concentration. Similarly it is unlikely that dNTP binding ($k_8$) has become rate-limiting since dNTP has been shown to be in rapid equilibrium with the enzyme-DNA complex for wild-type Klenow fragment, with an association rate ($k_2$) of at least 10$^7$ M$^{-1}$ s$^{-1}$ (Eger et al., 1991).

To investigate the relative rates of the conformational change ($k_7$) and phosphodiester bond formation ($k_8$) for the mutant proteins under consideration, the rate of addition of a single dTTP residue to poly(dA)-oligo(dT) ($k_4$) was compared to the rate of addition of dTTPαS ($k_9$) for each mutant protein. Fig. 3 (panel A) shows an experiment of this type for the D882E mutant protein. Because these experiments were carried out under conditions of enzyme excess, the rate of disappearance of the starting material could be fit to a single exponential (see Fig. 4), allowing the calculation of the apparent first-order rate constants, $k_6$ and $k_9$ (Table II). Using poly(dA)-oligo(dT), the mutant proteins R668A, Q849A, D882E, D882S, and E883A gave slower addition of dTTPαS. Essentially similar results were obtained using a natural DNA substrate (universal primer annealed to M13, Fig. 3, panel B), except that the magnitude of the sulfur elemental effect was different (Table II). Intriguingly, the mutant proteins D705A, D705S, D882A, and D882S did not extend the universal primer-M13 substrate. In case this observation reflected a problem specific to dGTP utilization by these proteins, the experiment was repeated using a series of natural DNA substrates where the required dNTP substrate was different. Again, no synthesis was observed (data not shown). It is unclear why these mutant proteins are capable of catalyzing the polymerase reaction on poly(dA)-oligo(dT) but not on other DNAs. Some obvious differences between the two substrates are the unusual structure of poly(dA)-oligo(dT) (Coll et al., 1987; Nelson et al., 1987; DiGabriele et al., 1989), the ability of the homopolymer to undergo slippage at the primer terminus, and the presence of a larger excess (in length terms) of single-stranded template in the M13-derived substrate.

The following controls demonstrated that the reactions on both DNA substrates were performed under pseudo first-order conditions for both sets of experiments. To demonstrate that the enzyme to DNA ratio of at least 90:1 was adequate to ensure single turnover conditions, the DNA concentration was reduced 10-fold in the reactions with the D882E, D882S, and Q849A mutant proteins and the rates of incorporation of dNTP and dNTPαS were shown to be unchanged. For those enzymes (D705A, D705S, and E710A) having higher $K_{mgdNTP}$ values, the reactions were repeated using a 4-fold higher concentration of dNTP or dNTPαS on the poly(dA)-oligo(dT) substrate. There was no change in the observed rates, confirming that the original reactions did indeed contain saturating levels of nucleotide. Although $K_{mgdNTP}$ is approximately the same as $K_{mgdNTPαS}$ for wild-type Klenow fragment (Burgers and Eckstein, 1979), we wanted to rule out the possibility that Q849A, D882E, and E883A showed an elemental effect with dNTPαS because they did not bind the thionucleotide substrate well. We therefore measured the addition of dGTPαS to the universal primer-M13 substrate.

![Fig. 4. Rate of addition of dGTPαS to the universal primer-M13 substrate catalyzed by the Q849A protein. The amount of starting material remaining is plotted as ln(%primer remaining) versus time. The slope of the line gives the parameter $k_8$ (Table II).](image)

| Table II |
|------------------|-----------------|----------------|------------------|----------------|-----------------|-----------------|
| Protein         | $k_{mgdNTP}$ | $k_{mgdNTPαS}$ | $k_{mgdNTP}/k_{mgdNTPαS}$ | $k_{mgdNTP}$ | $k_{mgdNTPαS}$ | $k_{mgdNTP}/k_{mgdNTPαS}$ | $k_{mgdNTP}$ | $k_{mgdNTP}$ | $k_{mgdNTPαS}$ | $k_{mgdNTP}/k_{mgdNTPαS}$ |
| Wild type       | >25'          | >25            | 1.6               | >25           | >25            | 1.6               | >25           | >25            | >25             | 1.6               |
| R668A           | 0.38          | 0.054          | 7.0               | 7.5           | 2.3            | 3.2               |               |                |                 |                  |
| D705A           | 0.0027        | 0.0010         | 2.7               | <0.0005       |                |                   |               |                |                 |                  |
| D705S           | 0.0083        | 0.0027         | 3.1               | <0.0005       |                |                   |               |                |                 |                  |
| E710A           | 4.1           | 1.5            | 2.7               | 25            | 5.3            | 6                 | 2.3           | 2.7            |                 |                  |
| N845D           | 12            | 5.1            | 2.3               | >25           | >25            | 21                | 6             | 2.3           | 2.7             |                   |
| Q849A           | 13            | 0.36           | 35                | 18            | 0.84           | 21                | 7             | 1.3           | 21              |                   |
| H881A           | 16            | 6.2            | 2.6               | >25           | 4.6            | >6.4              | 10            | 2             | 5               |                   |
| D882A           | 0.053         | 0.038          | 1.4               | <0.0005       |                |                   |               |                |                 |                  |
| D882E           | 0.0079        | 0.00056        | 14                | 0.090         | 0.00063        | 140               |               |                |                 |                  |
| D882S           | 0.011         | 0.0013         | 8.1               | <0.0005       |                |                   |               |                |                 |                  |
| E883A           | 15            | 0.53           | 29                | 19            | 0.28           | 69                | 3-5.5         | 0.25-0.45`    | 12               |                   |

* Values are the average of two or more determinations. The rates measured in separate experiments were in good agreement (typically 2-fold).

* The substrate was 5' end-labeled oligo(dT)$_5$ annealed to poly(dA).

* $k_{mg}/k_{mgαS}$ is the ratio of the observed rate of primer extension with dNTP to the observed rate of primer extension with dNTPαS.

* The substrate was the synthetic 13/20-mer described previously (Kuchta et al., 1987).

* Values of $k_{mg}$ in excess of 7 min$^{-1}$ have considerable error because of the speed of the reaction. Rates greater than 25 min$^{-1}$ could not be measured manually. (This consideration does not apply to the burst rate data in the last three columns.)

* Reported by Miirahi et al. (1985) for the same substrate.

* Because of the slower reaction with this mutant protein, an acceptable fit of the data could be obtained over a range of rates, as indicated.
for these mutant proteins using a 4-fold higher concentration of dGTPαS. Again, no changes in reaction rates were observed.

The above experiments were performed with a mixture of dNTPαS diastereomers although Klenow fragment only uses the Sp diastereomer (Burgers and Eckstein, 1979). Using the pure Sp diastereomer of dTTPαS, k₄ was measured for Q849A and E883A on poly(dA)-oligo(dT) and the rates of the reaction were found to be no different than when using the mixture. This result indicated that the incorrect (Rp) diastereomer, which binds more weakly to the enzyme and is not incorporated into DNA (Burgers and Eckstein, 1979), did not affect the reaction rates, consistent with our expectations based on the 10-fold difference in binding constants between the Sp and Rp diastereomers.

Quenched-flow Studies—For some of the mutant proteins in this study (E710A, Q849A, H881A, and E883A), the pre-steady-state rates measured manually on the poly(dA)-oligo(dT) and universal primer-M13 substrates had considerable errors because of the speed of the reactions. Additional analysis of these proteins was therefore carried out in a quenched-flow apparatus using a synthetic oligonucleotide substrate. With DNA in excess over enzyme, the resulting time course showed biphasic kinetics (Fig. 5), with the initial rapid burst of product formation giving the rate of the reaction up to and including phosphodiester bond formation. The sulfur elemental effects, calculated from the burst rates at saturating nucleotide concentrations, were in good agreement with the manually determined values (Table II).

The biphasic character of the time course indicated that, for these mutant proteins and the 13/20-mer substrate, the rate-limiting step in the steady-state follows the chemical step. When using oligonucleotide substrates, such as the 13/20-mer, on which the reaction is limited to addition of a single dNTP residue, the steady-state rate for wild-type Klenow fragment is limited by the rate of dissociation of the DNA product (Kuchta et al., 1987). Assuming this same step is rate-limiting for the mutant proteins, their DNA dissociation rates can be derived from the slow phase of the time course (Table I); the values are largely consistent with the K_D values determined by DNase I protection. While the observation of a slow step subsequent to the chemical step for these four mutant proteins on the 13/20-mer substrate raises the possibility (mentioned earlier) that a similar situation may apply to the homopolymer substrate used in the steady-state and single turnover experiments, it is important to note that the two substrates are not strictly comparable. The polymerase reaction on the 13/20-mer is limited to addition of a single nucleotide, whereas multiple rounds of processive nucleotide addition can take place on the homopolymer. (Indeed, the rate-limiting step for wild-type Klenow fragment is different in the two situations, being dominated by the conformational change (k₄) under circumstances that permit processive synthesis (Mizrahi et al., 1985).) For the four mutant proteins under consideration, the comparison may, however, be valid since the processivity of all four mutant proteins was extremely low (Polesky et al., 1990). Consistent with this reasoning, there is broad agreement between the k₄ values, derived from the slow phase of the reaction with the 13/20-mer (Table I), and the steady-state k₄ values (on the homopolymer) for the E710A, H881A, and E883A proteins. (By contrast, wild-type Klenow fragment, which exhibits substantial processivity on the homopolymer substrate, has a k₄ 40-fold faster than k₄.) For the Q849A mutant protein, however, k₄ is 100-fold lower than k₄, suggesting that there may be important differences in the behavior of this protein in the two assay systems, such that its rate of recycling appears to be 100-fold slower on the homopolymer than on the oligonucleotide substrate.

A further complexity in the quenched-flow data was that the slow phase of the time course showed an unexpected dependence on dNTP concentration. This was most pronounced for the H881A mutant protein but was detectable for all four mutant proteins studied. While we do not fully understand the reason for the dNTP dependence of the slow phase for the mutant proteins but not for wild-type Klenow fragment, it can be interpreted kinetically in terms of a dNTP-assisted dissociation of the product DNA from the enzyme, according to the following scheme:

\[
E + 13/20-mer \rightleftharpoons E + 14/20-mer + dATP \\
\]

\[
\rightleftharpoons E + 14/20-mer + dATP \rightleftharpoons E + 14/20-mer + dATP \]

Fitting of the rate data to this scheme gave apparent K₄ values for the dNTP dependence of the slow phase ranging from 15 to 38 μM for the four mutant proteins. These values seem reasonable given that the dATP that becomes bound to the incorrect (Rp) diastereomer of dTTPαS, whereas multiple rounds of processive nucleotide addition can take place on the homopolymer. (Indeed, the rate-limiting step for wild-type Klenow fragment is different in the two situations, being dominated by the conformational change (k₄) under circumstances that permit processive synthesis (Mizrahi et al., 1985).) For the four mutant proteins under consideration, the comparison may, however, be valid since the processivity of all four mutant proteins was extremely low (Polesky et al., 1990). Consistent with this reasoning, there is broad agreement between the k₄ values, derived from the slow phase of the reaction with the 13/20-mer (Table I), and the steady-state k₄ values (on the homopolymer) for the E710A, H881A, and E883A proteins. (By contrast, wild-type Klenow fragment, which exhibits substantial processivity on the homopolymer substrate, has a k₄ 40-fold faster than k₄.) For the Q849A mutant protein, however, k₄ is 100-fold lower than k₄, suggesting that there may be important differences in the behavior of this protein in the two assay systems, such that its rate of recycling appears to be 100-fold slower on the homopolymer than on the oligonucleotide substrate.

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FIG. 5. Rapid-quench analysis of the polymerase reaction catalyzed by the E883A mutant of Klenow fragment. The reaction was carried out as described under “Experimental Procedures,” using 120 nM E883A protein, 600 nM [5'³2P]13/20-mer, and dATP, at concentrations of 10 (○), 30 (△), or 50 (■) μM, or dATPαS, at concentrations of 10 (□), 30 (Δ), or 50 (○) μM. Each time point represents the average of two measurements, whose values differed by no more than 3%. The simulated lines were fit to the scheme described under “Results” using the rate and equilibrium constants in Tables I and II.

DISCUSSION

Characterization of Mutations at Asp⁷⁰⁵, Glu⁷¹⁰, and Glu⁸⁶³—

To identify the amino acids which catalyze the polymerase reaction, we have extended our study of mutations within the proposed DNA-binding cleft of the polymerase domain. In our previous mutagenesis study, we identified a group of mutations at residues Arg⁷⁰⁵, Glu⁷¹⁰, and Asp⁸⁶³ which resulted in a large decrease in k₄. We have introduced new mutations at Asp⁷⁰⁵, Glu⁷¹⁰, and Glu⁸⁶³ whose side chains extend into the cleft and are close to Asp⁸⁶³ in the refined tertiary structure (Fig. 1). All of the residues that we have studied within this spatially defined region are highly conserved in other poly-

3 A. H. Polesky, unpublished data.
4 L. S. Beese and T. A. Steitz, unpublished data.
5 A. H. Polesky, unpublished data.
erase sequences (Delarue et al., 1990), suggesting that they may play important roles in substrate binding and catalysis.

To elucidate the contribution of individual amino acids to either substrate binding or catalysis, the new mutant proteins (D705A, D705S, E710A, E710D, and E883A) were first analyzed by steady-state kinetics. \( K_{\text{M(NTP)}} \) is thought to provide a good measure of \( K_{\text{M(NTP)}} \) in the ternary complex because of the rapid equilibration of dNTP with the enzyme-DNA binary complex (Bryant et al., 1983; Kuchta et al., 1987; Eger et al., 1991). Mutations at positions 705 and 710 cause an increase in \( K_{\text{M(NTP)}} \). Interestingly, the \( K_{\text{M(NTP)}} \) for E710D is about half of that for E710A, suggesting that an important interaction with the substrate may have been preserved by the more conservative Glu→Asp change. As in our previous study (Polesky et al., 1990), the \( K_m \) changes we have observed, although small, were reproducible. It remains an open question whether the enzyme-dNTP interaction is mediated by a large number of relatively weak interactions, or whether more dramatic effects on \( K_m \) will result from mutation of some other, as yet untested, side chain.

The increase in \( K_{\text{M(NTP)}} \) for mutations at Asp705 and Glu710 extends the group of mutations defining the putative dNTP-binding site, Tyr706, Arg741, and Asn860, further into the cleft (Fig. 1) and closer to mutations which affect \( k_{\text{cat}} \). In fact, mutations at position 705, in addition to their effect on \( K_{\text{M(NTP)}} \), also cause a substantial decrease in \( k_{\text{cat}} \). It is not surprising for a particular amino acid side chain within an active site to participate in both substrate binding and catalysis. The residue might interact with one of the substrates in both the ground state and the transition state, or, in the case of a multifunctional side chain, the residue could play a direct role both in catalysis (for example, as a general acid or general base) and in substrate binding.

The new mutations at positions 705, 710, and 883 all cause an increase in \( K_{\text{M(DNA)}} \) of 5–30-fold. As noted in our previous study, almost all mutations within the cleft affect the activity of the enzyme for DNA, suggesting that the DNA substrate makes multiple interactions within the cleft. The decrease in DNA affinity caused by mutations in the carboxylates Asp705, Glu710, and Glu883 contrasts with our previously reported increase in DNA affinity caused by several substitutions at Asp705 (Table I (Polesky et al., 1990)). The Asp705 result could be rationalized by postulating that these mutations remove a sulfur elemental effect suggests that phosphodiester bond formation has become at least partially rate-limiting for this E710D (see below). The E710A mutant protein also shows a relatively modest decrease in \( k_{\text{cat}} \) relative to wild-type. In the absence of additional evidence, as for E883A, there is no compelling reason to propose that Glu710 is directly involved in catalysis. This interpretation seems compatible with the almost negligible effect on \( k_{\text{cat}} \) of the more conservative E710D mutation; it could be argued that even a small change in the position of a side chain that is intimately involved in catalysis should have a dramatic effect on \( k_{\text{cat}} \) (compare, for example, the effect of mutation to Asp of the catalytic Glu860 in triosephosphate isomerase; Raines et al., 1986). The E710D protein is intriguing, as an example of a mutant protein where the correlation between \( k_{\text{cat}} \) and \( K_{\text{M(DNA)}} \) breaks down. Since, as indicated above, Glu710 is likely to be part of a metal ion-binding site, a possible explanation is that, under the saturating DNA conditions of our assay, the binding of DNA to the E710D protein, together with the negative charge preserved by this particular mutation, permits normal binding of Mg\(^{2+}\) and allows the reaction to take place at close to wild-type rates. By contrast, the \( K_{\text{M(DNA)}} \) result suggests that in the free protein, the E710D mutation might be too early in the cleft to effectively destabilize the metal ion-binding site.

The observation that the E710D protein shows a similar processivity to that of wild-type Klenow fragment, in spite of its higher \( K_{\text{M(DNA)}} \), implies that, for this mutant protein, the \( K_{\text{M(DNA)}} \) measured on the binary enzyme-DNA complex may underestimate the DNA affinity in an actively synthesizing ternary complex.

Use of \( \alpha\)-Thio-substituted dNTPs to Identify the Rate-limiting Step of the Polymerase Reaction—By comparing the rate of addition of the first nucleotide with the rate of addition of the first two nucleotides, we have shown that the rate-limiting step for mutants of Klenow fragment with substitutions at Arg860, Asp705, and Glu883 occurs at or before phosphodiester

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bond formation. For the other proteins being studied (E710A, N845D, Q849A, H881A, and E883A), the forward reaction was too fast to allow an unambiguous interpretation of this experiment, although, as discussed above, the rapid-quench experiment suggests that DNA dissociation may be rate-limiting for E710A, Q849A, H881A, and E883A. Using α-thiol-substituted dNTPs to probe those steps of the reaction up to and including phosphodiester bond formation, we have demonstrated that the Q849A, D882E, D882S, and E883A mutant proteins show an elemental effect while N845D, D705A, D706S, E710A, and D882A do not. For R668A (and perhaps also H881A), the elemental effect is small and somewhat variable, being detectable on only one of the DNA substrates used. The simplest interpretation of the data is that the polymerase reaction involves two steps between the enzyme-DNA-DNTP and enzyme-DNA-PP, ternary complexes, one of which is slowed by sulfur substitution while the other is not. For those enzymes that show an elemental effect, the substitution-sensitive step is partially or completely rate-limiting, while, for those enzymes that do not show an elemental effect, the other step is rate-limiting, just as for wild-type Klenow fragment.

What do these two steps correspond to in physical terms? Clearly, one must be the chemical step in which the phosphodiester bond is formed. In order to interpret the phosphorothioate data one needs to be able to predict the magnitude of the change in the rate of the chemical step resulting from sulfur substitution. This is not a straightforward task. Model studies in free solution indicate a gradation of elemental effects in going from phosphate triesters, which show a rate decrease of 10- to 100-fold on substitution of sulfur at a nonesterified position (Heath, 1956a, 1956b), to diesters, in which sulfur substitution causes a more modest rate decrease (Herschlag et al., 1991), and finally monoesters, in which sulfur substitution actually increases the reaction rate (Breslow and Katz, 1968). These effects, which mirror the transition from an associating (S∞2) displacement for triesters, to a largely dissociative mechanism for monoesters, are attributed to the lower electronegativity of sulfur compared with oxygen.

Before the recent study of Herschlag et al. (1991) on phosphodiesterases, elemental effect data have frequently been interpreted based on the expectation, from the triester studies, of large elemental effects in the chemical step of a reaction. Thus, the observation of a slower reaction rate with a thioester compared with a phosphate ester has frequently been taken as evidence that the chemical step of an enzyme-catalyzed reaction is rate-limiting, provided that the K∞ values for the two substrates are the same (Eckstein, 1975). For wild-type Klenow fragment, where K∞(dNTP)TP is the same (for the Sp diastereomer) as K∞(dNTP)P (Burgers and Eckstein, 1979), the lack of a significant elemental effect (1.6-fold) on the pre-steady-state rate of the polymerase reaction was the basis of the proposal that a nonchemical step, described as a conformational change (k0), is rate-limiting (Mizrahi et al., 1988). A similar mechanism has been proposed for T7 DNA polymerase (Patel et al., 1991). Since a diester arguably provides a better model for the polymerase reaction than a triester, a smaller elemental effect would now be predicted, subject to the obvious caveats that the enzyme active site itself may modify the nature of the transition state and that effects other than electronegativity have been ignored.

Our revised expectations as to the magnitude of the sulfur elemental effect necessitate re-examination of the conclusions from earlier studies. First, are there indeed two steps between formation of the enzyme-DNA-dNTP ternary complex and completion of phosphodiester bond formation? Since one cannot assume a large elemental effect in the chemical step (k4) of the reaction, one might argue that the existence of the preceding slow step (k3), inferred from the experiments of Mizrahi et al. (1985), is not proven. However, subsequent studies, as well as the present work, confirm the existence of two steps, since a sulfur elemental effect can be observed in some circumstances (wild-type Klenow fragment with incorrect dNTPs, Ruchta et al. (1988); Q849A and E883A mutant proteins in this study) but not in others (e.g. wild-type Klenow fragment with correct dNTPs, Mizrahi et al. (1985)). Independent evidence obtained from pulse-chase and pulse-quench studies, and not involving sulfur elemental effects, demonstrates conclusively that, for wild-type Klenow fragment, the chemical step of the polymerase reaction is flanked by nonchemical steps (Aahlberg and Benkovic, 1991), validating the conclusions made by Mizrahi et al. (1985).

Second, which of the two steps is slowed by phosphoro-thioate substitution? The large elemental effects observed in some of our experiments, together with the results of Herschlag et al. (1991), suggest that the elemental effect in our system may have contributions from factors other than electronegativity differences. Thus, it is conceivable that either the chemical or the nonchemical step could be sensitive to sulfur substitution. Given that the data of Aahlberg and Benkovic (1991) prove that, for wild-type Klenow fragment under single turnover conditions, the rate-limiting step is nonchemical, the lack of an elemental effect on the forward reaction (Mizrahi et al., 1985) shows that this nonchemical step is insensitive to sulfur substitution, allowing us to deduce that the chemical step (k4) is the one probed by the elemental effect.

The extremely complicated elemental effect data from the mutant derivatives of Klenow fragment (Table II) may offer some clue as to the basis for the elemental effect on the two DNA substrates (Table II), although the nucleophile (the 3'-hydroxyl of dTMP) and the leaving group (PPi) are the same for both (the substrate is trivially altered, dGTP in one case and dTTP in the other). It is also hard to explain why three different mutations at one position (D882E, D882S, and D882A) result in markedly different elemental effects, although the k∞ values for the three mutant proteins are very similar (Table I).

A more coherent explanation for all the above observations can be made if one takes into account the additional steric constraints imposed by substitution of sulfur for oxygen. Not only is sulfur significantly larger than oxygen (van der Waal's radius 1.85 Å versus 1.4 Å, but the sulfur-phosphorus bond is longer than the oxygen-phosphorus bond (1.94 Å versus 1.51 Å) (Eckstein, 1975). In an enzyme active site where space may be limited, the increased size of the phosphorus-

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7 In a much earlier study (Eckstein, 1968), the rates of alkaline hydrolysis of a cyclic phosphodiester and its phosphorothioate analog suggest a similar trend.

8 The bond lengths for the oxygen-phosphorus and sulfur-phosphorus bonds are from the crystal structures of cytidine 2',3'-cyclic phosphate and uridine 2',3'-O,cyclic phosphorothioate, respectively.
sulfur group could be a major influence on the rate of the reaction. An analogous situation, where sulfur substitution on a protein (by replacement of Ser with Cys) has a dramatic effect on activity, indicates the potential magnitude of steric effects (McGrath et al., 1989; He and Quiocho, 1991).

Our dNTPαS results can be explained using steric considerations in the following way. In the ground state the nucleotide substrate binds to the polymerase active site with the same affinity regardless of thio phosphorothioate substitution, as shown by Burgers and Eckstein (1979). However, in the transition state for phosphodiester bond formation there is a steric clash between the enzyme and the sulfur atom which slows the rate of the corresponding step of the reaction. The variable elemental effects resulting from the different substitutions at Asp842 can readily be explained by noting the correlation between the magnitude of the elemental effect and the size of the mutant amino acid side chain (D882E→D882S→D882A). This correlation implies that the steric clash involves (at least in part) the Asp842 side chain in wild-type Klenow fragment and is relieved by substitution of the smaller Ala, and augmented by substitution of Ghu. If correct, this explanation leads to an important conclusion, namely that Asp842 must be positioned extremely close to the α-phosphate position in the transition state for the chemical step of the polymerase reaction.

Throughout the above discussion of steric effects on dNTPαS incorporation, we have made the implicit assumption that the contribution of electronegativity (discussed previously) to the magnitude of the sulfur elemental effect is small (consistent with the data of Herschlag et al., 1991), although more complicated situations, in which the observed effect has both steric and electronegativity components, can also be imagined. The following reasoning suggests that this assumption is correct. If all the mutations at Asp842 do indeed cause the chemical step, which is sensitive to sulfur substitution, to become rate-limiting, then the lack of an elemental effect in the reaction catalyzed by the D882A mutant protein (in which we have proposed that the steric constraints are alleviated) could indicate that the electronegativity of the α-phosphorus is not an important factor in the polymerase reaction.

The steric model can also be used to explain the differences in the magnitude of the sulfur elemental effect with the two DNA substrates. Considering the structural differences between poly(dA)-oligo(dT) and natural DNA (Coll et al., 1987; Nelson et al., 1987; DiGabriele et al., 1989), it seems reasonable to suppose that there may be subtle differences in the positioning of the 3'-hydroxyl within the cleft in the two cases, resulting in different degrees of steric interference in the transition state. Variable positioning of the primer terminus might also account for the different relative effects seen with each mutant protein on the two substrates, either by influencing the degree to which the sulfur-sensitive step is rate-limiting on the two substrates, or because the mutations themselves allow subtle changes in the position of the DNA and influence the degree of steric interference in rather unpredictable ways.

In the explanation presented above, the sulfur elemental effect, although likely to be largely steric in origin, remains a probe for the chemical step of the polymerase reaction. Therefore many of the conclusions drawn from our data and from previous results are the same. Thus, the lack of an elemental effect for wild-type Klenow fragment still leads to the conclusion that the slowing of the sulfur-sensitive step by the thionucleotide substitution is masked by an even slower preceding step. Similarly, the large elemental effects resulting from the Q849A and E883A mutations can most simply be attributed to a slowing of the chemical step by these mutations so that it is no longer kineticlly invisible. (The assumption that all the observed elemental effects can be traced back to the same steric clash, manifested in the chemical step with the wild-type enzyme, is the most economical way of accommodating the data in this and the previous studies. Clearly other explanations are possible, but they suffer from the disadvantage of requiring one to postulate that each circumstance in which an elemental effect is observed has caused a subtle rearrangement of the active site region in the ternary complex so as to introduce a new steric clash.)

An important consequence of the steric argument that we have presented is that the observation of a significant elemental effect for a mutant protein can only be interpreted unambiguously, as indicating that the chemical step is rate-limiting, when the mutation decreases the size of the amino acid side chain (as for Q849A and E883A). When the altered side chain is larger than in the wild-type protein one cannot rule out the possibility of additional steric constraints in the reaction of the mutant protein. Analogously, one must interpret with caution the lack of an elemental effect for any mutant protein in which the mutant amino acid has a smaller side chain than the corresponding wild-type residue. Perhaps the substitution of a smaller side chain may have relieved the steric clash and abolished the sulfur elemental effect even though the chemical step is still rate-limiting. This is certainly a question that can legitimately be raised concerning Asp842, where the effects of mutations, both in vivo and in steady-state experiments, are quite dramatic and show some parallels with mutations at Asp842. Moreover, sequence alignments suggest that Asp842 and Asp843 are universally conserved in all known polymerase sequences (Delarue et al., 1989), implying that both contribute in some important way to the polymerase reaction. The alternative interpretation, that Asp842 plays a crucial role in the nonchemical step of the reaction, is, of course, equally consistent with our data. Although there is no physical description for the nonchemical step, one should not underestimate its potential mechanistic importance; as suggested previously (Bryant et al., 1983; Kuchta et al., 1987) it might serve as an initial editing step that involves hydrogen-bonding of the dNTP to the template strand, leading to a ternary complex poised for catalysis (thus explaining the observation that the initial binding of dNTP is fairly insensitive to the identity of the template base). The proximity of Asp842 to the site of nucleophilic substitution can be further explored by making mutations that increase the size of the side chain, and these experiments are in progress.

The Identity of Active Site Residues and Possible Models for Catalysis of the Polymerase Reaction—The results of our steady-state analysis of mutant Klenow fragment derivatives suggest an important role in catalysis of the polymerase reaction for the side chains of Arg870, Asp842, Gln871, and Asp872. Furthermore, the dNTPαS experiments implicate Gln871 and...
Glu$^{68}$ (and maybe Arg$^{68}$ and His$^{68}$) in the chemical step of catalysis. The steric arguments outlined above suggest that Asp$^{705}$ may be close to the $\alpha$-phosphate position in the transition state, and leave open the possibility that Asp$^{705}$ may also be close. The failure of the D882A mutant protein to show an elemental affect is attributed to relief of the steric clash by substitution of the smaller side chain and does not necessarily rule out a role for Asp$^{705}$ in the chemical step. Indeed, the elemental affect data for the series of mutations at Asp$^{782}$ can most simply be explained on the assumption that these mutations affect the chemical step, and it could also be argued that the proximity of Asp$^{782}$ to the site of nucleophilic displacement makes a catalytic role for this residue a likely possibility.

Sequence comparisons between polymerases (Delarue et al., 1990) give us confidence that our mutagenesis experiments to date have explored most of the active site region, and that the important active site residues will be drawn from these amino acids or their close neighbors. Two sequence motifs (A and C in the nomenclature of Delarue et al. (1990)) are common to the entire polymerase family of DNA- and RNA-dependent DNA and RNA polymerases. On the Klenow fragment sequence these motifs are centered on Asp$^{782}$ and Asp$^{705}$, both of which are invariant throughout the compared sequences, as noted above. Preliminary work on other polymerases confirms the importance of the residue equivalent to Asp$^{782}$. When the amino acid residues corresponding to Asp$^{782}$ and Glu$^{68}$ are mutated in $\varepsilon$29 and herpes simplex virus DNA polymerases, the mutations result in a substantial loss of activity in crude assays (Bernad et al., 1990; Dorsky and Crumpacker, 1990).  

How, then, might the proposed active site residues contribute to achieve catalysis of the polymerase reaction, a nucleophilic attack on the $\alpha$-phosphate of the dNTP by the 3'-hydroxyl at the primer terminus (Atkinson et al., 1969; Kornberg, 1969)? Although other explanations are possible, the observation that the reaction proceeds with inversion of configuration about the $\alpha$-phosphate (Brody and Frey, 1981) suggests an associative $S_{N}2$ mechanism with a sequence transition state or intermediate. It is likely that there is substantial transition state stabilization by a variety of side chain contacts (see e.g. Pauling (1946) and Fersht (1987)), given the involvement of a considerable number of side chains in catalysis, the overlap between DNA or dNTP binding and catalytic functions for the majority of these side chains, and the participation of side chains such as Arg$^{698}$ and Gln$^{690}$ that are unlikely, on chemical grounds, to play a “classical” role in catalysis. However, transition state stabilization may not be the only factor contributing to catalysis. Attack by the 3'-hydroxyl of a DNA molecule is likely to require deprotonation either by a general base or as a result of coordination to a metal ion. The extremely low values of $k_{cat}$ resulting from mutations at Asp$^{782}$ and Asp$^{705}$ make these residues, rather than Glu$^{18}$, His$^{68}$, or Glu$^{68}$, the most likely candidates for a catalytic general base. This idea is also consistent with the conservation of these Asp residues in other polymerase sequences (Delarue et al., 1990). However, the residual levels of activity seen in the D705A and D882A mutant proteins (at least on the homopolymer substrate) may argue against either of the Asp residues serving as a general base, unless, of course, another side chain (or a water molecule) participates when the true catalytic side chain is absent, as has been seen in other systems (Stayert et al., 1990; Komives et al., 1991).

A further possibility which, in our view, deserves serious consideration, is that one or more divalent metal ions play a significant role in catalysis of the polymerase reaction. The ubiquity of divalent metal ions in phosphoryl transfer reactions and their possible catalytic functions have been discussed in several reviews (see e.g. Mildvan and Loeb (1979) and Knowles (1980)). In addition to the Mg$^{2+}$ which is believed to be chelated to the $\beta$- and $\gamma$-phosphates of the incoming dNTP (Sloan et al., 1975; Burgers and Eckstein, 1979; Mullen et al., 1990), a metal ion could function to stabilize negative charge on the phosphate oxygens in the pentacovalent transition state or intermediate. This type of stabilization has been suggested for the phosphoryl transfer reactions catalyzed by staphylococcal nuclease (Cotton et al., 1979) and DNAse I (Suck and Oefner, 1986) both of which use a general base to promote the initial nucleophilic attack. A second group of enzymes, exemplified by alkaline phosphatase (Kim and Wyckoff, 1991), the 3',5'-exonuclease of Klenow fragment (Freemont et al., 1988; Beece and Steitz, 1991), and perhaps even ribozymes (Freemont et al., 1988; Dange et al., 1990; Perreault et al., 1991), carry out catalysis using an appropriately positioned pair of metal ions. Polarization of the nucleophile by one metal ion assists in deprotonation and thus facilitates nucleophilic attack. A second metal ion stabilizes negative charge on the phosphoryl oxygens in the transition state or intermediate, and may also facilitate departure of the leaving group. As pointed out by Beece and Steitz (1991), a mechanism of this type is a very real possibility for the polymerase reaction. In this case, one enzyme-bound metal ion would be coordinated to the 3'-hydroxyl at the DNA primer terminus, while a second metal ion would be located close to the $\alpha$-phosphate position. The existence of divalent metal ion sites within the cleft on the Klenow fragment structure, as suggested by our DNA binding data and visualization of the pentacovalent intermediates (Beece and Steitz, 1989), is tantalizing, as is the obvious analogy with the 3',5'-exonuclease active site on the other domain of Klenow fragment (Joyce, 1991), where the major catalytic players are proposed to be two divalent metal ions anchored by four carboxylate side chains (Freemont et al., 1988; Beece and Steitz, 1991; Derbyshire et al., 1991).

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A third sequence motif (B) is conserved only in DNA-dependent DNA and RNA polymerases. From evidence obtained with Klenow fragment and herpes simplex virus DNA polymerase, this motif, containing an invariant Lys and a very highly conserved Tyr and Gly (Lys$^{782}$, Tyr$^{766}$, and Gly$^{767}$ in Klenow fragment), is likely to be involved in dNTP binding (Basu and Modak, 1987; Larder et al., 1987; Gibbs et al., 1988; Mullen et al., 1989; Polesky et al., 1990; Rush and Konigsberg, 1990; Carroll et al., 1991).
Side chains involved in catalysis of the polymerase reaction of DNA polymerase I from Escherichia coli.
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