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A Domain of the Klenow Fragment of *Escherichia coli* DNA Polymerase I Has Polymerase but No Exonuclease Activity

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ABSTRACT **The Klenow fragment of DNA polymerase I from *Escherichia coli* has two enzymatic activities: DNA polymerase and 3′–5′ exonuclease. The crystal structure showed that the fragment is folded into two distinct domains. The smaller domain has a binding site for deoxynucleoside monophosphate and a divalent metal ion that is thought to identify the 3′-5′ exonuclease active site. The larger C-terminal domain contains a deep cleft that is believed to bind duplex DNA. Several lines of evidence suggested that the large domain also contains the polymerase active site. To test this hypothesis, we have cloned the DNA coding for the large domain into an expression system and purified the protein product. We find that the C-terminal domain has polymerase activity (albeit at a lower specific activity than the native Klenow fragment) but no measurable 3′–5′ exonuclease activity. These data are consistent with the hypothesis that each of the three enzymatic activities of DNA polymerase I from *E. coli* resides on a separate protein structural domain.**

Key words: protein domain, polymerase, 3′–5′ exonuclease, artificial gene, expression vector

INTRODUCTION

DNA polymerase I (Pol I) of *Escherichia coli* was the first template-directed DNA-polymerizing enzyme to be discovered and provides a simple model for the study of the enzymatic reactions involved in DNA replication. The two major roles of Pol I in *E. coli* are the repair of damaged duplex DNA and the processing of Okazaki fragments into high molecular weight DNA.[1] To achieve these functions Pol I, which is a monomer of 103,000 daltons, possesses three enzymatic activities: DNA polymerase, 3′–5′ exonuclease implicated in editing out mismatched nucleotides, and 5′–3′ exonuclease.[1] Limited proteolysis of whole Pol I cleaves the molecule into two fragments.[2,3] The larger C-terminal fragment (Klenow fragment; mol. wt. 68,000 daltons) has both the DNA polymerase and 3′–5′ exonuclease activities whereas the smaller N–terminal fragment (mol. wt. 35,000 daltons) has only the 5′–3′ exonuclease activity.[3] The DNA coding for the Klenow fragment has been cloned into an expression vector[4] that produces large quantities of protein.

The crystal structure of the Klenow fragment has shown that the molecule is folded into two distinct domains.[5] Interpretation of this structure in terms of biochemical data suggested that the active sites for the 3′–5′ exonuclease and the polymerase activities might be located on separate domains. The smaller domain (N-terminal 200 residues) forms a complex with deoxynucleoside monophosphate (dNMP) and a bound metal ion. Kinetic evidence[6] and the weak amino acid sequence homologies between this domain and the 3′-5′ exonuclease subunit of DNA polymerase III[7] have suggested that the dNMP binds at the 3′-5′ exonuclease active site.

The larger domain (C-terminal 400 residues) forms a deep cleft of the appropriate dimensions to bind a B-DNA double helix.[5] Support for this location of the DNA binding site comes from electrostatic calculations[8] and from the positions of two point mutations, *polA5* and *polA6*, which affect the interaction of the enzyme with DNA.[5,9] The high degree of amino acid sequence homology between Pol I and T7 DNA polymerase in those polypeptides forming the surface of the cleft suggests that the large domains of these enzymes have evolved from a common precursor and that the cleft has an important function.[10] Model building of DNA onto the protein identified a plausible position for the 3′ terminus on the large domain and raised the possibility that the large domain contains the active site for polymerization.[5] This idea was supported by affinity labeling of the dNTP binding site and by DNA footprinting experiments which allowed us to deduce a position for the primer terminus.[7]

To test the hypothesis that the large domain of the Klenow fragment contains the polymerase but not the 3′-5′ exonuclease active site, the DNA coding for the large domain (residues 515–928 on the Pol I sequence[11]) has been cloned into an expression vector under the control of the strong leftward promoter (P_L) of phage lambda. Using this plasmid clone we have overproduced and purified the isolated C-terminal domain and examined its enzymatic properties in vitro.

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MATERIALS AND METHODS

Materials

Restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim. T4 DNA ligase and poly[d(A-T)] were from Boehringer Mannheim. [α-^{32}P] dATP (>400 Ci/mmol) was from Amersham. All other deoxyribonucleotides were from Pharmacia. Guanidine·HCl, lysozyme, and bovine serum albumin were from Sigma. DEAE-Sephacel and Sephacryl S-200 were from Pharmacia. The Klenow fragment of Pol I was purified from an overproducing strain as described previously.[4]

Construction of a Plasmid Which Overproduces the C-Terminal Domain of Klenow Fragment

The 1,515-base-pair *Xho*II fragment, extending from position 1,542 on the *polA* sequence[11] to position 3,057 beyond the end of the gene,[12] was purified by agarose gel electrophoresis. The DNA was isolated by electroelution and chromatography on a minicolumn (approx. 100 μl) of DEAE-cellulose (Whatman DE-52). The *Xho*II fragment was ligated into the *Bam*HI site of the expression vector pASl.[13] After transformation of strain CJ109 (a λcI^+ lysogen[4]) to ampicillin resistance, plasmids were prepared by the alkaline lysis procedure.[14] Digestion with *Eco*RI and *Sac*I (which cuts asymmetrically within the *Xho*II fragment) identified a plasmid, pCJ120, having the inserted DNA in the correct orientation.

Growth and Induction of Bacteria

The plasmid pCJ120 was introduced into the host AR120, which carries a λcI^+ cryptic prophage.[15] The resulting strain was grown at 37°C in 10 liters of Maximal Induction Medium[15] containing 1% (w/v) glucose and 100 μg/ml ampicillin. Nalidixic acid was added to the culture to 40 μg/ml when the OD_{650} reached 1.0, and incubation was continued for an additional 6–12 hours. The cells were harvested by centrifugation and stored frozen.

Purification of the C-Terminal Domain

All steps were carried out at 0–4°C unless otherwise stated. Cells (2.5 g) were thawed and resuspended in 25 ml of 50 mM Tris·HCl (pH 7.5) containing 0.1 mM phenylmethylsulfonylfluoride (PMSF), 2.5 mM 2-mercaptoethanol, and 1 mg of freshly dissolved lysozyme per ml. After 20 min at room temperature, the mixture was cooled on ice and sonicated briefly to complete lysis. On centrifugation at 1,000 g for 5 min, more than 95% of the C-terminal domain remained associated with the cell debris pellet. The supernatant was discarded and the cell debris was extracted with 10 ml of buffer A (20 mM Tris·HCl, pH 7.0/0.1 mM PMSF/2.5 mM 2-mercaptoethanol) containing 6 M guanidine· HCl and 1 M NaCl. A small amount of insoluble material was removed by centrifugation and the supernatant was then diluted with buffer A (30 ml) to give a final protein concentration of approx. 2 mg/ml. The C-terminal fragment was then renatured by successive dialyses against three changes of buffer A containing 1 M NaCl followed by three changes of buffer A alone. Any insoluble material appearing in the renaturation step was removed by centrifugation. The clarified solution was then applied to a DEAE-Sephacel column (1.5 cm × 14 cm). The column was washed with buffer A and the C-terminal domain was eluted with a linear gradient of 0–0.5 M NaCl in buffer A: the C-terminal fragment eluted at 0.15 M NaCl. Fractions were assayed for protein concentration and polymerase activity.

Preparative Gel Electrophoresis

Purified C-terminal domain from the DEAE-Sephacel column was loaded onto 12 lanes of a 15% SDS/polyacrylamide gel (approx. 30 μg per 0.8-cm slot). Electrophoresis was carried out until the bromophenol bulue marker dye had reached the bottom of the gel, after which one lane of the gel was excised and stained in Coomassie Brilliant Blue to locate the C-terminal domain. After destaining, the corresponding region of the unstained gel was cut out and the C-terminal domain was reconstituted according to the method of Weber and Kuter.[16]

Preparation of the C-Terminal Domain Without Denaturation

To obtain some of the large domain in a soluble form, AR120 cells containing pCJ120 were induced with nalidixic acid as described above and harvested after only 2 hours induction. Cells (6.5 g) were lysed in 24 ml of buffer, essentially as described above. On centrifugation the bulk of the large domain sedimented with the cell pellet and was discarded; however, a portion (10–20%) remained soluble. Nucleic acids were removed from the soluble fraction by gentle mixing for 1 hour at 4°C with 20 ml of DEAE-cellulose (Whatman DE-52) in 20 mM Tris·HCl, pH 7.0. The resulting supernatant was fractionated by ammonium sulfate precipitation. The fraction precipitating between 60% and 85% saturation (at 0°C) was shown by gel analysis to contain the C-terminal domain. This material was dissolved in 2 ml of 100 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol, and 1 ml was applied to a column (1 cm × 48 cm) of Sephacryl S-200 in the same buffer. Fractions were assayed for polymerase activity and were examined by gel electrophoresis. The amount of large domain in the peak fractions was estimated by comparison with known quantities of Klenow fragment or large domain on the same gel.

Polymerase Assay

The C-terminal fragment was assayed by the standard DNA polymerase assay,[17] with poly[d(A-T)] as the template. One unit of polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 3.3 nmol of dNTP into trichloroacetic-acid-insoluble material in 10 min at 37°C.

3′-5′ Exonuclease Assay

The labeled assay substrate was prepared from *E. coli* chromosomal DNA digested to completion with *Sau*3AI. Digested DNA (24 μg, approx. 290 pmol of ends) was 3′ end-labeled by using 1 unit of Klenow fragment in a 50-μl reaction containing 10 nmol unlabeled dGTP and 30 pmol [α-^{32}P]dATP (3,000 Ci/nmol) for 10 min at room temperature. Excess unlabeled dATP (1 nmol) was added and incubation was continued for a further 1 min to ensure that all the 3′ ends were extended to the same extent (leaving a 2-nucleotide 5′ extension). The reaction was terminated by addition of EDTA to 20 mM and the Klenow fragment was inactivated by heating at 70°C for 15 min. The labeled DNA was phenol extracted, passed through a 1 ml column of Sephadex G-50 to remove unincorporated nucleotides, and recovered by ethanol precipitation.

The assay reaction contained 3′ end-labeled DNA (approx. 4×10^{-7} M ends) in 6 mM Tris·HCl, pH 7.5, 6 mM $MgCl_2$, 6 mM 2- mercaptoethanol, and 50 mM NaCl. Enzyme was added and the mixture was incubated at 37°C. Samples were removed at intervals and the DNA was precipitated in a microfuge tube by addition of trichloroacetic acid (to 5% w/v) in the presence of bovine serum albumin (0.5 mg/ml) as carrier. The radioactivity in both supernatant and pellet was determined by Cerenkov counting with an appropriate correction for the difference in quenching.

Other Methods

The procedure for SDS/polyacrylamide gel electrophoresis was according to Laemmli.[18] Protein concentrations were determined by the Bradford method[19] with crystalline bovine serum albumin as a standard.

RESULTS

Overproduction and Purification of the C-Terminal Domain

In order to express the C-terminal domain of the Klenow fragment, we used the expression vector pAS1,[13] which carries the strong leftward promotor (P_L) of phage λ, followed by the ribosome binding site and the translational initiation codon of the cII gene. The ATG codon is immediately followed by a *Bam*HI restriction site into which we inserted a *Xho*II fragment encoding the C-terminal 414 amino acids of the *PolA* structural gene. The position of the *Xho*II site was such that simple ligation of the complementary *Xho*II and *Bam*HI cohesive ends maintained the correct translational reading frame. The translated product should have the amino-terminal sequence Met-Asp-Leu-Gln...., where the Asp residue corresponds to position 515 of the Pol I sequence, [11] and is located close to the C-terminus of helix F on the Klenow fragment three-dimensional structure (Fig. 1).

The resulting plasmid, pCJ120, was introduced into the strain AR120, which carries the wild-type λ repressor and *N* gene on a defective prophage.[15] Expression from the P_L promoter on pCJ120 was initiated by the addition of nalidixic acid to induce the SOS response in the bacterial host. Gel electrophoresis of a whole cell lysate after induction showed a band of the predicted size (46,000 daltons), amounting to 20–30% of the total cell protein (Fig. 2, lanes 1–3). This band was not present in a control lysate from AR120 containing the vector plasmid, pAS1 (data not shown).

Cells for protein purification were typically induced for 10 hours. Unfortunately the C-terminal domain resulting from this overproduction protocol was present in an insoluble form and sedimented with the cell debris after cell lysis. We were unable to extract the domain by addition of nonionic or zwitterionic detergents or high concentrations of NaCl or ammonium sulfate. In order to solubilize the C-terminal domain we had to use denaturation with 6 M guanidine·HCl followed by renaturation of the extracted protein (see Materials and Methods). The insolubility of the domain resulted in substantial purification so that the renatured protein was at least 85% homogeneous, as judged by SDS/polyacrylamide gel electrophoresis (Fig. 2, lane 4). However, the extracted domain, though soluble, still appeared to aggregate and this caused difficulties in subsequent chromatographic stages. Gel filtration on Sephacryl S-200 resulted in elution of the C-terminal domain at the void volume, indicating an apparent molecular weight in excess of 200,000 daltons. Ion-exchange chromatography on DEAE-Sephacel produced a small peak of nearly homogeneous protein comigrating with a peak of polymerase activity (Fig. 3). The recovered material corresponded to 2% of the total protein loaded onto the column but had 60% of the polymerase activity. This suggests that the bulk of the C-terminal domain was inactive, perhaps due to failure to form a completely native structure on renaturation, and may have aggregated or precipitated on the column. The purified C-terminal domain from the DEAE-Sephacel column (typically 0.1–0.2 mg from 2.5 g of cells) was judged to be at least 95% pure by SDS/polyacrylamide gel electrophoresis (Fig. 2, lane 5).

Since we intended to measure the polymerase activity of the C-terminal domain, we wanted to include a fractionation step based on molecular size. In this way, we could be sure that the 46,000-dalton domain was not contaminated by any of the endogenous polymerases, all of which are substantially larger. As described above, gel filtration was unsatisfactory, presumably due to aggregation of the domain. We therefore fractionated a portion of the purified material on a preparative SDS/polyacrylamide gel. The 46,000-dalton domain was cut out from the gel and the protein reconstituted as described by Weber and Kuter.[17]

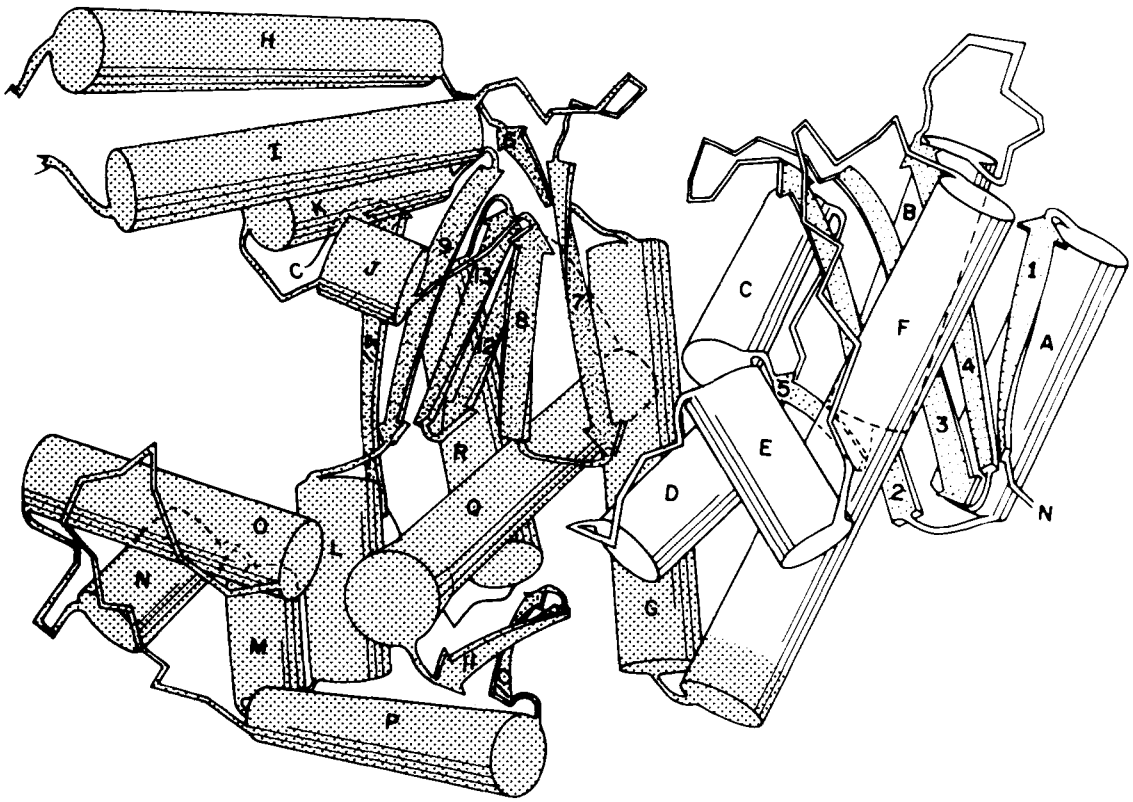

Fig. 1. Tertiary structure of Klenow fragment (for details, see reference 5). The cylinders represent α-helices and the arrows represent β-sheet. The shaded region indicates the portion of the Klenow fragment sequence expressed in the C-terminal domain overproducer clone.

Enzymatic Activity of the C-Terminal Domain

Assays of the peak fraction from the DEAE-Sephacel column showed that the purified domain had polymerase activity, but the specific activity was only 2% of that of native Klenow fragment. The gel-purified material was also found to have polymerase activity, although two-fold lower than the column-purified fragment. The peak fraction from the DEAE-Sephacel column was also assayed for 3′-5′ exonuclease activity (Fig. 4). Because of the difference in polymerase specific activities, we compared amounts of Klenow fragment and C-terminal domain that would give comparable rates of polymerization. We found that the C-terminal domain showed no detectable 3′-5′ exonuclease activity over a 24-hour period, whereas Klenow fragment catalyzed rapid removal of the 3′ terminal-labeled nucleotide. Using dilutions of Klenow fragment, we showed that exonuclease activity would easily have been detectable at 1% of this level (Fig. 4). Thus the exonuclease: polymerase ratio for the domain is considerably less than 1% of the value for whole Klenow fragment.

Preparation of the Large Domain Without Denaturation

We were concerned that the low polymerase specific activity of the C-terminal domain could have been caused by the denaturation-renaturation protocol. Denaturation and renaturation of Klenow fragment from 6 M guanidine·HCl showed recovery of 60–70% of the original polymerase activity and 90–95% of the 3′-5′ exonuclease activity, implying that the active site regions of Klenow fragment can refold correctly on renaturation. However, the possibility remains that the domain structure may be less stable and may not fold correctly.

We therefore extracted the large domain by a procedure that did not involve denaturation. Our aim was not to purify the domain to homogeneity, but merely to fractionate it sufficiently to remove contaminating cellular polymerases. With a shorter induction time, and therefore a lower level of expression, a small amount of the C-terminal domain could be recovered in soluble form in the cell extract. The crude material was treated with DEAE-cellulose to remove nucleic acids and then fractionated (and concentrated) by ammonium sulfate precipitation. Gel filtration on Sephacryl S-200 gave a peak of polymerase activity that coincided with the C-terminal domain peak, located by gel electrophoresis. There was no detectable large domain eluting at the void volume; a small amount of polymerase activity at the void volume was probably due to whole Pol I. A parallel experiment with Klenow fragment as an internal marker showed that Klenow fragment migrated

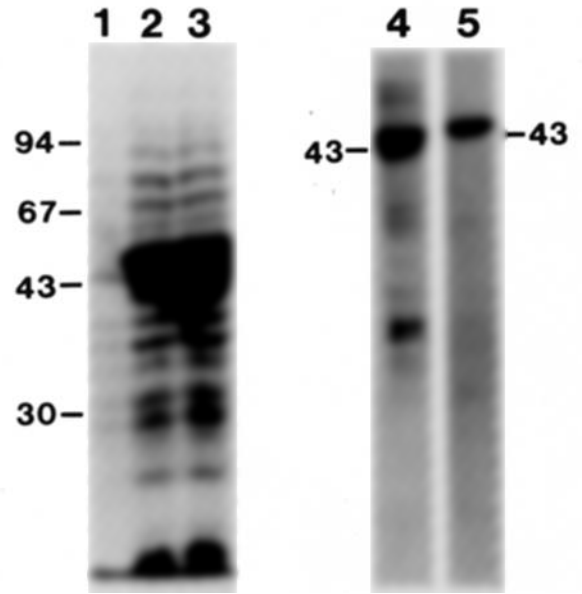

Fig. 2. Overproduction and purification of the C-terminal domain of Klenow fragment. Lanes 1–3 show the analysis on a 12% SDS/polyacrylamide gel of samples taken during the growth and induction of strain AR120 containing pCJ120. Lane 1, whole cell lysate before the addition of nalidixic acid; lane 2, after 4.5 hours incubation with nalidixic acid; lane 3, after 9 hours incubation with nalidixic acid. The positions of marker proteins, and their molecular weights (in kilodaltons) are indicated on the left. Lanes 4 and 5 show electrophoresis on a 15% SDS/polyacrylamide gel of samples taken during the purification procedure. Lane 4, material obtained after extraction of the cell pellet with 6 M guanidine·HCl and subsequent renaturation of the extract; lane 5, peak fraction after chromatography on DEAE-Sephacel. For each lane, the position of a 43,000-dalton marker, run on a parallel lane of the same gel, is indicated.

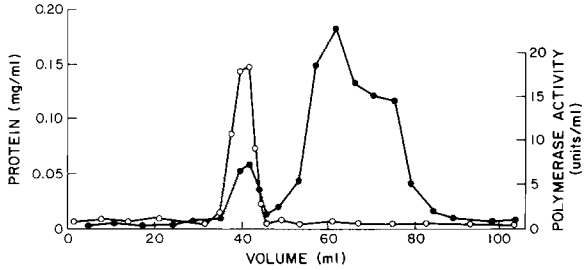

Fig. 3. Elution of the C-terminal domain of Klenow fragment from a column of DEAE-Sephacel, as described in Materials and Methods. Polymerase assays were carried out in triplicate on selected fractions. ●, protein concentration; ○, polymerase activity.

several fractions ahead of the large domain. Thus the domain was well separated from the larger cellular polymerases and from Klenow fragment, a potential breakdown product of Pol I.[2] After gel filtration the C-terminal domain was still quite impure; in the peak fraction the domain was one of seven major components. (Because of the large number of impurities, it was impossible to measure the exonuclease activity of the domain prepared in this way.) We estimated the amount of the C-terminal domain in the peak fractions by comparison with a series of known quantities of Klenow fragment on the same gel. Using this value, the calculated polymerase specific activity (in units/mg) of the domain was 10% of that of the whole Klenow fragment.

The tenfold-lower polymerase activity of the C-terminal domain can largely be accounted for by alterations in K_m for the DNA and dNTP substrates. The K_m for poly[d(AT)] is about seven-fold higher for the domain than for Klenow fragment (Fig. 5). Similarly, the K_m for dNTP utilization is increased about fivefold over the Klenow fragment value of 18 μM (data not shown). Addition of glycerol to 12% had no effect on Klenow fragment; however, it caused the polymerase activity of the large domain to increase almost fivefold (i.e., to nearly 50% of the Klenow fragment specific activity). Glycerol may facilitate the interaction with substrates either directly or by stabilizing the structure of the domain. A rough calculation assuming Michaelis-Menten kinetics predicts, on the basis of the changes in K_m alone, that the large domain should have 17% of the specific activity of whole Klenow fragment under standard assay conditions. This value agrees quite well with our experimental observations and therefore argues against the possibility that a significant proportion of the large domain preparation was inactive when isolated without denaturation. The close agreement also suggests that, aside from K_m, other kinetic constants as turnover number are similar for the domain and whole Klenow fragment.

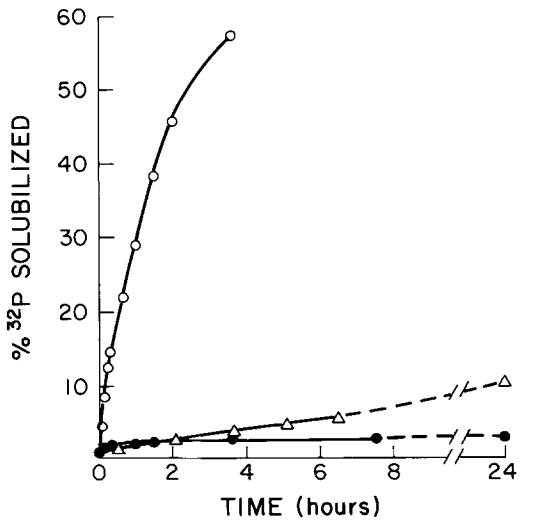

Fig. 4. Exonuclease activity of intact Klenow fragment and of the C-terminal domain. The 3′-5′ exonuclease activity was measured by the release of acid-soluble radioactivity from DNA labeled at the 3′ end with [^{32}P]. The acid-soluble [^{32}P] is expressed as a percentage of the total radioactivity in each sample. Reactions contained the following amounts of enzyme, expressed in terms of polymerase activity units: ○, 3×10^{-3} units Klenow fragment; △, 3×10^{-5} units Klenow fragment; ●, 4×10^{-3} units C-terminal domain.

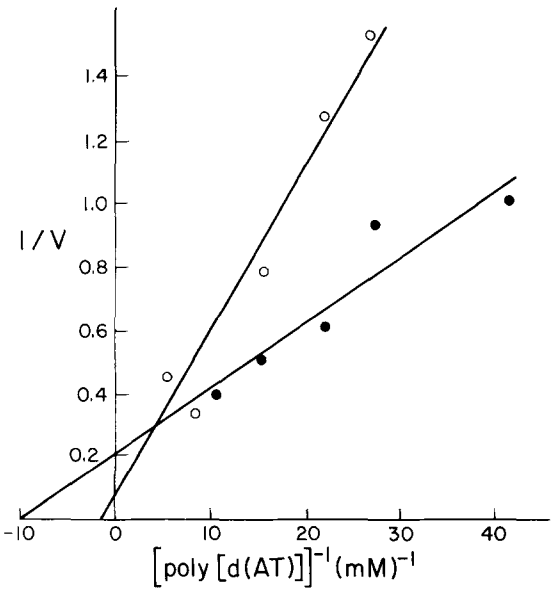

Fig. 5. K_m determination for poly[d(AT)] utilization by Klenow fragment and its C-terminal domain. The rate of polymerization was determined as a function of poly [d(AT)] concentration, expressed as molarity of total nucleotides. The reaction mixture was the same as for the standard polymerase assay except that the concentration of poly[d(AT)] was varied between 2.4 × 10^{-5} M (0.16 A_{260} units/ml) and 1.9 × 10^{-4} M (1.24 A_{260} units/ml). Using approx. 0.08 units of enzyme, the amount of incorporation of [α^{32}P]dATP into polynucleotide in 10 min gave a measure of the polymerization rate. Each measurement was carried out in triplicate. The double-reciprocal plot was fitted by a least-squares regression analysis. ●, Klenow fragment; ○, C-terminal domain. The horizontal intercepts gave K_m values of 10^{-4}M for Klenow fragment and 6.6 × 10^{-4} M for the C-terminal domain. (The poly[d(AT)] concentration in the standard assay is 1.3 × 10^{-4}M.)

DISCUSSION

To test the hypothesis that the two active sites of Klenow fragment are separated, with the polymerase site alone on the C-terminal doman, we have constructed a plasmid that directs expression of large quantities of this domain. Purification of the domain was complicated by the insolubility of the overproduced protein. Similar problems have been reported in other systems.[20,21] In such cases, denaturation appears to be the only way of solubilizing the overproduced protein but, once renatured, the protein usually remains soluble for subsequent manipulations. Our observations suggest that the C-terminal domain of Klenow fragment may be slightly destabilized by the absence of the other domain and that this may affect its ability to refold correctly on renaturation. This could account for the tendency of the renatured C-terminal domain to behave during subsequent purification stages as if a portion of it were aggregated (and enzymatically inactive). Because of these difficulties, we carried out assays both with large domain purified to homogeneity using the denaturation/renaturation protocol, and with partially purified large domain that had been extracted in a soluble form without denaturation.

Our results show that the C-terminal 400 residues of Klenow fragment (the large domain) have DNA polymerase activity but no measurable 3′-5′ exonuclease activity. Hence, the active site for polymerization must lie on the large domain. Although the small domain is clearly dispensable for the polymerization reaction, its presence enhances the polymerase activity. Thus, under standard assay conditions, the polymerase activity of Klenow fragment is some tenfold greater than that of the large domain. The difference is largely, if not completely, attributable to changes in K_m for the two substrates. A plausible explanation is that the small domain stabilizes the large domain structure so that, in its absence, there is an unfavorable entropy contribution to substrate binding. This idea is also consistent with our observation that glycerol in the reaction may compensate partially for the lack of the small domain, and with our observations during the purification procedure (discussed above).

The conclusion that the large domain contains the active site for polymerization was also supported by earlier experiments.[7] Photoaffinity labeling suggested that the deoxynucleoside triphosphate binding site is close to the side chain of Tyr 766, located at the C-terminus of helix O in the large domain (Fig. 1). The results of DNA footprinting experiments, when interpreted in terms of the model of the Klenow fragment with DNA, indicated that the primer terminus of the DNA substrate must be located on the large domain, and are compatible with a position for the primer terminus close to helix O.

The absence of measurable 3′-5′ exonuclease activity on the large domain is consistent with (but does not prove) our hypothesis that the small domain contains the active site for the 3′-5′ exonuclease activity. These data alone do not rule out the possibility that the small domain contains some but not all of the residues essential for the 3′-5′ exonuclease active site while the large domain contains the remaining residues. Nor do they exclude the possibility that the 3′-5′ exonuclease active site actually lies on the large domain but becomes unstable when the small domain is removed. However, we consider the latter two alternatives less likely in view of other data.

The strongest argument suppporting the idea that the small domain contains the 3′-5′ exonuclease active site is based on the location of the dNMP binding site and its functional significance. Kinetic data have shown that nucleoside 5′-monophosphates are competitive inhibitors of the 3′-5′ exonuclease but do not affect the polymerase reaction.[6] (This implies not only that the dNMP binds at the 3′-5′ exonuclease active site but also that the polymerase and exonuclease active sites are functionally separate.) The crystal structure shows that Klenow fragment contains a single binding site for deoxynucleoside monophos-

phate located on the small domain.[5] Moreover, the contacts between the dNMP molecule and the protein are consistent with existing data on the requirements for binding and inhibition by dNMP analogs.[1,6] A relationship between the dNMP binding site and the 3′-5′ exonuclease activity is also suggested by the weak amino acid sequence homologies between the small domain of Klenow fragment and epsilon, the 3′-5′ exonuclease subunit of DNA polymerase III. The homologous regions surround the dNMP binding site, and residues that ineract with the dNMP molecule tend to be conserved.[7] Further evidence for the location of the 3′-5′ exonuclease active site comes from work with a mutation that was introduced into Klenow fragment to test the hypothesis that residues surrounding the dNMP binding site are involved in the exonuclease reaction. The resulting protein, in which Asp 424 has been changed to Ala, has normal polymerase activity but altered 3′-5′ exonuclease activity, implying that our location for the 3′-5′ exonuclease active site is correct and that the two active sites are remote from one another and largely independent (V. Derbyshire, C.M. Joyce and N.D.F. Grindley, unpublished work). The different response of the two enzymatic functions to heat treatment (with the 3′-5′ exonuclease being the more labile) also implies that the catalytic sites are structurally independent, so that the disruption of one does not affect the other.[22]

The proposed organization of Klenow fragment (and of whole Pol I) with each enzymatic activity on a separate domain is clearly analogous to the subunit structure of DNA polymerase III in which the polymerase and 3′-5′ exonuclease activities are located on independent subunits.[21]

The available data, both from our own work and from that of others, leads us to conclude that the active sites for the two enzymatic functions of the Klenow fragment lie on separate structural domains. The only result that conflicts with this conclusion is a nuclear magnetic resonance experiment indicating that the distance between the nucleoside monophosphate site and a spin-labeled ATP analog is only about 7 Å.[23] One possible explanation for the disagreement is that the bulky spin-label may have caused the nucleotide analog to bind not only to the dNTP site (as was inferred from a competition experiment) but also to another site close to the nucleoside monophosphate. Because of the extreme distance dependence of paramagnetic spin relaxation effects, any binding at the dNTP site on the large domain would not have been observable in this experiment. Alternatively, the calculations may not have completely eliminated contributions from the interaction between the two nucleotides in free solution.

Our current model for the Klenow fragment structure has the polymerase and exonuclease active sites separated by 20–30 Å. Is this separation compatible with the requirements of the enzymatic reaction? Pol I catalyzes the incorporation into DNA of about 700 nucleotides per minute at 37°C.[1] Simultaneous turnover of dNTPs to the corresponding dNMPs amounts to about 10% of the level of incorporation.[24] Thus, on average, there should be about one hydrolysis event per second, involving movement of the primer terminus from the polymerase active site to the exonuclease active site and back again. Recent data from Mizrahi and Benkovic[25] demonstrated unambiguously that such movement can take place without dissociation of the DNA from the enzyme. Given the rate of sliding of *lac* repressor on DNA (10^6 nucleotides/second[26]), it seems that the primer terminus could slide between the polymerase and exonuclease active sites at a rate that is extremely fast relative to the rate of nucleotide addition. Thus, there is no reason to expect that this separation of active sites would impede the DNA synthesis reaction.

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