Highly selective affinity labeling of the primer-binding site of *E.coli* DNA polymerase I

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Received 26 July 1990

Highly selective affinity labeling of the primer site of *E.coli* DNA polymerase I was performed with the 5'-reactive derivatives of oligothymidylate in the presence of poly(dA) template. Subtilysine cleavage proved that the site of affinity modification belonged to the 'Klenow' part of DNA polymerase I. If taken separately, Klenow fragment was not labeled by these oligonucleotide derivatives. The sites of affinity labeling were tested in the structure of DNA polymerase I by hydroxylamine cleavage. At least two sites of labeling were revealed. The main one was localized between Gly-833 and His-928.

E.coli DNA polymerase I; Klenow fragment; Affinity labeling

1. INTRODUCTION

The method of highly selective affinity labeling based upon 'catalytic competence' of covalently bound protein and initiating substrate was successfully applied to the studies of active centers of DNA-dependent RNA polymerases [1-5]. It is interesting to extend this approach to the analysis of other template-dependent enzymes. The most suitable object of such a study is *E. coli* DNA polymerase I because an extensive X-ray analysis of its Klenow fragment has been recently performed [6-8].

A scheme of highly selective affinity labeling of DNA polymerase is shown in Fig. 1. Enzyme-template complex is treated with an affinity reagent which is analogous to the initiating substrate and carries reactive groups (X) at the 5'-end of oligonucleotide. This results in the covalent binding of the affinity reagent residues both inside and outside the active center. These residues happen to be 'catalytically' competent in some of the molecules of the modified enzyme: addition of an appropriate radioactive dNTP results in their elongation by a radioactive nucleotide due to the catalytic action of the active center.

This study seems to be the first to present the results on highly selective labeling of *E. coli* DNA polymerase I. The localization of the modified amino acid residues of this enzyme is also analyzed.

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Abbreviation: KF, Klenow fragment

2. MATERIALS AND METHODS

2.1. E. coli *DNA polymerase I was obtained as in [9]*

Klenow fragment of DNA polymerase I was kindly donated by Dr S.Ch. Degtyarev (NIKTI BAV, USSR)

2.2. *Synthesis of tri- and tetrathymidylate derivatives*

The structures of these reagents are shown in Fig. 1. The synthesis of derivatives of a similar type was described earlier [10]. The alkaline groups of these compounds can be activated by $NaBH_4$ reduction of aldehyde groups under soft conditions. Products were homogeneous as judged by HPLC. Two absorption maxima were observed for reagent I: 265 nm, 347 nm, $D_{265}/D_{347} = 1.04$, $\lambda_{min} = 300$ nm; and for reagent II: 268 nm, 347 nm, $D_{268}/D_{347} = 1.43$, $\lambda_{min} = 302$ nm. After $NaBH_4$ reduction $D_{347} = 0$.

The oligonucleotides used were synthesized and kindly donated by Dr T.W. Abramova.

2.3. *Affinity labeling of* E. coli *DNA polymerase I and KF*

The reagent (10^{-4} M) was added to the reaction mixture (10 μl) containing 0.05 M Hepes/NaOH pH 8; 2.5×10^{-3} M $MgCl_2$; 2.5×10^{-4} M EDTA; 5×10^{-2} M KCl; poly(dA)$_{20-200}$ 0.5 OD_{260}/ml; 5×10^{-3} M NaF and 1-2 μg of the enzyme. The mixture was incubated for 30 min at 37°C followed by the addition of $NaBH_4$ (0.01 M) and reincubation for 60 min. Subsequently [α-^{32}P]dTTP (spec. act. >111 PBq/mol, 7×10^{-7} M) was added and the mixture was stored at 37°C for 15 min. The reaction was stopped by the addition of 2.5 μl of the denaturing mixture: 5% SDS; 5% β-mercaptoethanol; 50% glycerol and 0.1% Bromophenol blue. The mixture was heated at 56°C for 10 min and electrophoresed in a Laemmli system [11] in 8% acrylamide. The proteins were stained with R-250 Coomassie brilliant blue, the dried gels were subjected to autoradiography.

2.4. *Cleavage of the affinity labeled DNA polymerase I*

Cleavage by subtilysine was carried out according to Klenow et al. [12].

2.5. *Cleavage of the modified DNA polymerase I by hydroxylamine [13,14]*

After the affinity labeling of *E. coli* DNA polymerase I 1% SDS and 1% β-mercaptoethanol were added followed by incubation of the

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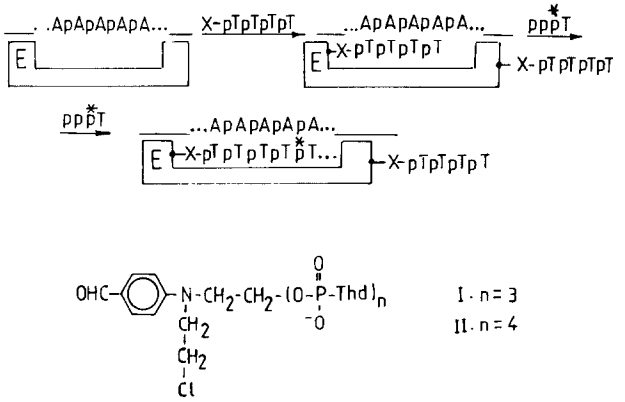

Fig. 1. Scheme of highly selective affinity labeling of DNA polymerase. *p = radioactive phosphorus atom; X-pTpTpTpT = affinity reagent; X = residue of affinity reagent covalently bound with the enzyme; E = enzyme.

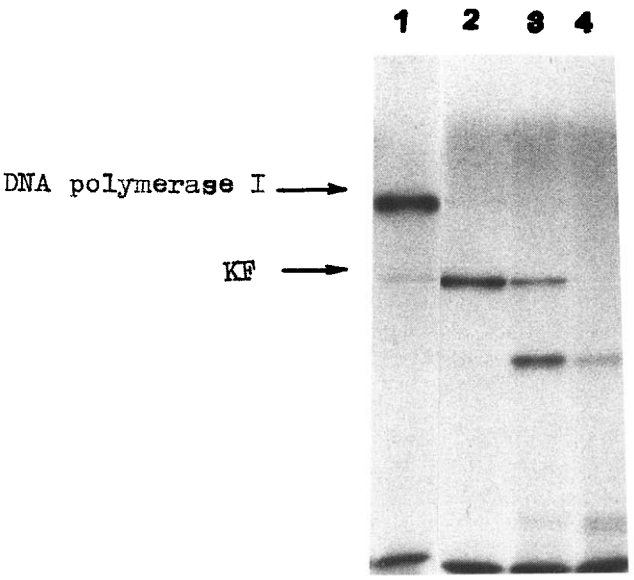

Fig. 3. Cleavage of the affinity labeled DNA polymerase I by subtilysine. Modification of the enzyme and its cleavage by subtilysine were performed using reagent II as described in section 2. Radioautograph of gel electrophoresis of the affinity labeled DNA polymerase I before cleavage (1) and after its cleavage by subtilysine of 3×10^{-4} mg/ml (2), 3×10^{-3} mg/ml (3), 3×10^{-2} mg/ml (4).

mixture for 30 min at 37°C. Hydroxylamine (1 M in 0.1 M K_2CO_3, pH 10) was supplemented and the reaction proceeded for 2.5 h at 37°C.

3. RESULTS AND DISCUSSION

All experiments on the affinity labeling of DNA polymerase I were carried out in the presence of poly(dA) template. At the initial stage the complex of DNA polymerase I with poly(dA) was treated with one of the reagents (I, II). At the second stage [α-^{32}P]dTTP was added as an elongating substrate. Then the enzyme was denatured by SDS and the mixture was subjected to gel electrophoresis. The patterns obtained are shown in Fig. 2 (lanes 1,2). The intensity of labeling increased with the length of the oligonucleotide part of the reagents. This phenomenon is suggested to be related to the increase in the affinity of the primer to the DNA polymerase/template complex with the lengthening of the primer [15].

No labeling was observed in the presence of tetrathymidylate without a reactive group (Fig. 2, lane 4) or without $NaBH_4$ reduction (Fig. 2, lane 5). Reagents I and II have two reactive groups (aldehyde and alkylating) that are capable of modifying proteins. However, it is not clear as yet which of them provides

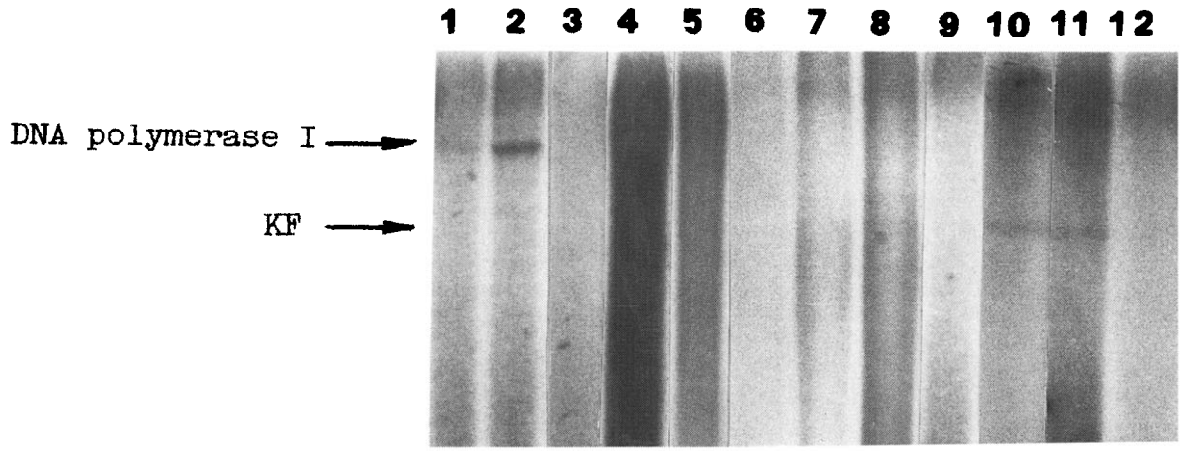

Fig. 2. Radioautograph of gel electrophoretic pattern obtained for DNA polymerase I and KF affinity labeled with reagents I and II. Affinity labeling of DNA polymerase I with reagent I (1), II (2), labeling of DNA polymerase I without reagent and template ('nonenzymatic labeling') (3); affinity labeling of DNA polymerase I without reagent, but in the presence of tetrathymidylate (4); with reagent II and without $NaBH_4$ (5); under conditions of preincubation with reagent II without template (6), without template and Mg^{2+} (7), without Mg^{2+} (8) (omitting compounds were added simultaneously with [α-^{32}P]dTTP); in the presence of noncomplementary poly(dC) template (9); labeling of KF without reagent and template ('nonenzymatic labeling') (10); affinity labeling of KF with reagents I (11) and II (12).

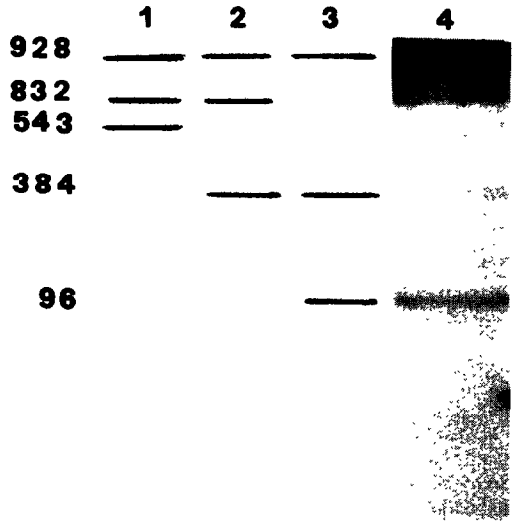

Fig. 4. Cleavage of the affinity labeled DNA polymerase I by hydroxylamine. Modification of the enzyme and its cleavage by hydroxylamine were performed using reagent II as described in section 2. Theoretical patterns of the arrangement of the cleavage products: the label is located to the left of Gly-544 (1), between Gly-544 and Asn-832 (2), to the right of Asn-832 (3); radioautograph of gel electrophoresis of the affinity labeled DNA polymerase I after its cleavage by hydroxylamine (4). Figures indicate the number of bases in peptides.

covalent binding in this case. We did not detect the affinity labeling of DNA polymerase I with reagent II under incubation without template (Fig. 2, lane 6), template and Mg^{2+} (Fig. 2, lane 7), or Mg^{2+} (Fig. 2, lane 8), the omitting compounds being added with [α-^{32}P] dTTP at the second stage. The enzyme was not labeled in the presence of noncomplementary template either (Fig. 2, lane 9).

Using compounds I and II as affinity reagents for KF, [^{32}P]incorporation in the protein was shown to be comparable with the level of 'nonenzymatic labeling' by [^{32}P]dNTP. This kind of labeling was reported earlier [16].

To localize the site of enzyme modification, we used the cleavage of the labeled DNA polymerase I by subtilysine, which resulted in the formation of KF [12], and the treatment of the modified protein by hydroxylamine. Cleavage by subtilysine showed the ^{32}P-label to be located in the Klenow part of DNA polymerase I (Fig. 3). This fragment carries the DNA polymerization site for binding of template, primer and dNTP. Besides, it has the 3′-5′-exonuclease active site.

Hydroxylamine cleavage of peptide bonds between asparagine and glycine was executed. DNA polymerase I has two such bonds: Asn-543–Gly-544 and Asn-832–Gly-833. Fig. 4 (lanes 1,2,3) shows theoretical patterns for localization of radioactive products by cleavage in the following ways: the label is (i) to the left of Gly-544; (ii) between Gly-544 and Asn-832; (iii) to the right of Asn-832. In fact, the label is included in the peptides Gly-544–Asn-832 and Asn-832–His-928 (Fig. 4, lane 4). At the same time, the results prove the most intensive labeling to occur at the peptide Asn-832–His-928.

Therefore, in this work for the first time we demonstrate that *E. coli* DNA polymerase I can be labeled in a template-dependent manner by reactive analogs of a complementary primer.

The results obtained suggest the 5′-end of the primer to be located closer to the region of contact between polymerase and exonuclease active sites [7]. The absence of the affinity labeling of KF in contrast with DNA polymerase I is likely to be due to the difference in spatial organization of functional contacts of the 5′-end of the primer with both the enzymes. Further application of this approach to DNA polymerases by using reactive derivatives of primers could shed light on the functional topography of DNA polymerases.

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