

EXCISION OF THYMINE DIMERS BY PROTEOLYTIC  
AND AMBER FRAGMENTS OF E. COLI  
DNA POLYMERASE I

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Received March 12, 1974

Summary

Excision of thymine dimers from specifically incised ultraviolet irradiated DNA by E. coli DNA polymerase I is stimulated by concurrent DNA synthesis. The 36,000 molecular-weight "small fragment" obtained by limited proteolysis of DNA polymerase I, which retains only the 5' → 3' exonuclease activity, also excises thymine dimers, but at one-tenth the rate of the intact enzyme. However, the rate of excision is increased by addition of the "large" 76,000-molecular weight fragment. With the further addition of the 4 deoxynucleoside triphosphates, permitting DNA synthesis to occur, excision approaches rates observed with the intact enzyme. The same result was obtained with a fragment of DNA polymerase I with 5' → 3' exonuclease activity that is present uniquely in polymerase I amber mutants.

Amber mutants in the structural gene for E. coli DNA polymerase I (pol A1), though grossly defective in their polymerase I activity, retain near normal levels of the 5' → 3' exonuclease activity that is part of the DNA polymerase I molecule (1). Since the 5' → 3' exonuclease activity in pol A1 mutants is associated with a polypeptide whose sedimentation coefficient (2.8S) is very similar to the "small fragment" with 5' → 3' exonuclease activity generated by proteolysis of polymerase I (2,3,4), it has been suggested that this activity represents the amber fragment itself, or more likely, a proteolytic cleavage product of it (1).

In exploring further the relationship of the pol A1 amber peptide to the small proteolytic fragment, we have inquired whether it can interact with the polymerase I "large fragment" that contains the polymerase and 3' → 5' exonuclease activities of the intact enzyme (2,3,4). For these studies, we have used as an assay for 5' → 3' exonuclease activity the

known capacity of the enzyme to promote thymine dimer excision from appropriately incised ultraviolet-irradiated DNA (5). We have found that the 2.8S peptide with 5' → 3' exonuclease activity present in pol A1 mutants of E. coli can interact with the polymerase I large fragment in a manner indistinguishable from the proteolytically generated small fragment. We have further observed that maximal rates of thymine dimer excision by polymerase I in vitro, and very likely in vivo, require the concerted action of both polymerase and 5' → 3' exonuclease.

#### Materials and Methods

Enzymes: The T4-induced UV endonuclease was purified, as described by Friedberg and King (6). It was stored at 4° in 10mM Tris-HCl (pH 8.0), containing 3% polyethylene glycol and 0.1mM EDTA. E. coli DNA polymerase I (fraction VII) was kindly provided by Dr. A. Kornberg. The small (36,000) and large (76,000) molecular-weight products of subtilisin cleavage of E. coli DNA polymerase I were a generous gift from Dr. Hans Klenow (University of Copenhagen). Partial purification of polymerase I-associated 5' → 3' exonuclease from extracts of E. coli pol A1 (JG 112), and the assay of DNA polymerase I and 5' → 3' exonuclease activity were performed as described by Lehman and Chien (1).

Incision of UV-Irradiated DNA: [<sup>3</sup>H] T7 DNA (5 to 10 x 10<sup>4</sup> cpm/μg) was prepared as described previously (6). Reaction mixtures for incision (1.0-1.5ml) contained 150-375 nmoles (nucleotide) of [<sup>3</sup>H]T7 DNA (irradiated at 3,000 ergs/mm<sup>2</sup>), 10mM EDTA and 300 to 1500 units of T4 UV endonuclease. Incubation was at 37° for 90 min. Based on a thymine dimer content of 1%, this amount of enzyme was in excess of that required to nick all dimer sites. Reactions were terminated by the addition of an equal volume of cold redistilled phenol (pH 7.0); the DNA was extracted twice with phenol and then dialyzed against 50 mM Tris-HCl (pH 8.0).

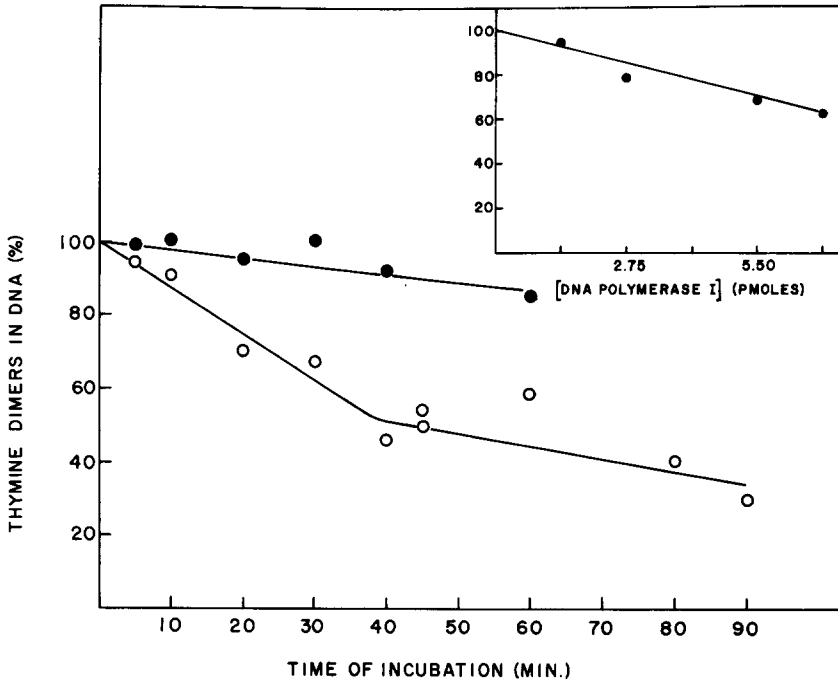


Figure 1. Kinetics of thymine dimer excision by DNA polymerase I. Details of the incubation and measurement of thymine dimers in DNA are described in the text. 1.4 pmoles enzyme ●; 6.9 pmoles enzyme ○. Inset figure shows the excision of dimers as a function of DNA polymerase concentration.

Excision of Thymine Dimers: Incubation mixtures (0.2ml) contained 10 nmoles (nucleotide) incised T7 DNA, 20mM Tris-HCl (pH 7.5) 20mM potassium phosphate (pH 7.5), 50  $\mu$ g/ml bovine serum albumin, 10mM N-ethylmaleiamide, 1.5  $\mu$ g *E. coli* tRNA, 7mM MgCl, and the amounts of enzyme indicated in the figure legends. Incubation was at 37°. When deoxynucleoside triphosphates (0.05M) were added, N-ethylmaleiamide was omitted. Reactions were terminated by the addition of 0.2ml of cold 10% trichloroacetic acid. After 15 min. at 0° the tubes were centrifuged at 5,000 xg for 15 min. and the content of thymine and thymine dimer in the acid-precipitable fraction was determined by formic acid hydrolysis, followed by two-dimensional thin-layer chromatography as described previously (7). Each experiment included a control sample of incised DNA incubated for 60 min. at 37° with an

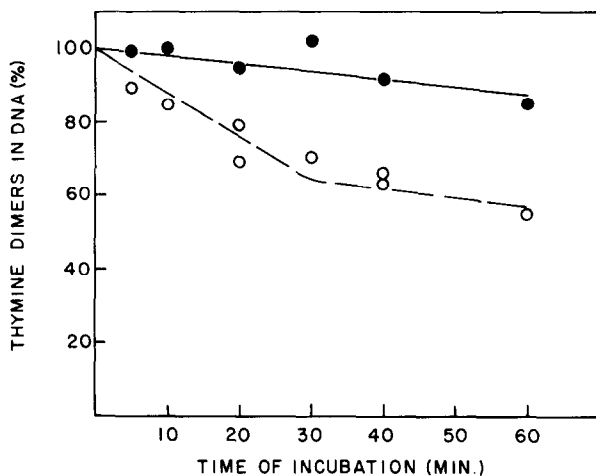


Figure 2. Effect of addition of deoxynucleoside triphosphates on rate of thymine dimer excision by DNA polymerase I. 1.4 pmoles of enzyme were used. No deoxynucleoside triphosphates ●; triphosphates added 0.

excess (130  $\mu$ g) of T2-induced thymine dimer excision exonuclease (8), to estimate the maximum extent of excision (80 to 90%). The control values were normalized to 100% and the data were corrected accordingly.

### Results

The excision of thymine dimers from incised irradiated T7 DNA by DNA polymerase I was linear with respect to both time and enzyme concentration (Fig. 1). As expected from the known stimulation of 5'  $\rightarrow$  3' exonuclease activity by concurrent polymerization (9), addition of the four deoxynucleoside triphosphates significantly increased the rate of thymine dimer excision (Fig. 2).

The rate of thymine dimer excision by the polymerase I small fragment which retains only the 5'  $\rightarrow$  3' exonuclease activity of the intact enzyme, was substantially less than that observed with the intact polymerase. Thus, approximately 10 times more small fragment than intact enzyme, estimated as molarity of polymerase protein, or on the basis of 5'  $\rightarrow$  3' exonuclease activity on the 5'- [ $^{32}$ P](dT) $_{300}$ .(dA) $_{5000}$  homopolymer pair, was required to produce the same rate of excision. However, addition of an equimolar

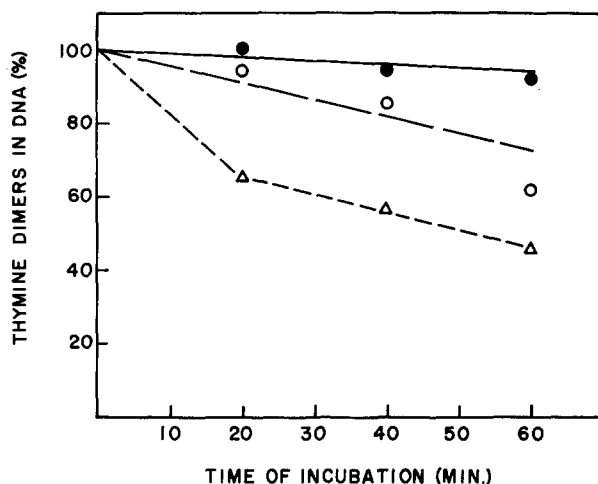


Figure 3. Thymine dimer excision by DNA polymerase I small fragment in the presence and absence of large fragment and deoxynucleoside triphosphates. Small fragment (7.7 pmoles) ●; small fragment (7.7 pmoles) plus large fragment (8.0 pmoles) ○; small fragment (7.7 pmoles) plus large fragment (8.0 pmoles) plus deoxynucleoside triphosphates ▲.

amount of the polymerase I large fragment, which bears the polymerase and 3' → 5' exonuclease activities increased the rate of thymine dimer excision by about 4-fold. In the presence of the four deoxynucleoside triphosphates the rate was increased even further (approximately 5-fold more) (Fig. 3).

A similar result was obtained with the 2.8S peptide containing the polymerase I-associated 5' → 3' exonuclease partially purified from a pol A1 amber mutant. As shown in Fig. 4, there was essentially no thymine dimer excision by an amount of 2.8S peptide equivalent to 7 pmoles of intact polymerase I as judged by 5' → 3' exonuclease activity on 5'-[<sup>32</sup>P](dT)<sub>300</sub>·(dA)<sub>5000</sub>. Addition of the large fragment resulted in a rate of excision comparable to that obtained with approximately 3 pmoles of intact enzyme, and this rate was further enhanced by addition of the four deoxynucleoside triphosphates.

Addition of deoxynucleoside triphosphates to either the small or large fragment alone did not significantly affect the rate of dimer excision. Furthermore, the differences in rates of dimer excision are not simply a reflection of changes in the distribution of dimers in acid-soluble and acid-insoluble oligonucleotides. Both the intact polymerase as well as the small

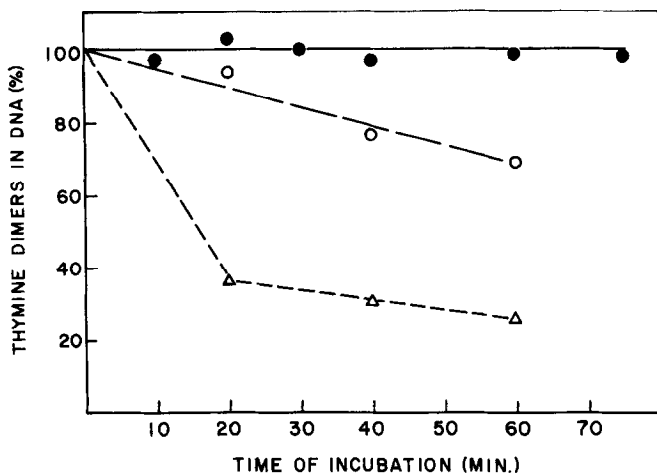


Figure 4. Thymine dimer excision by 2.8S peptide from *E. coli* pol A1. 2.8S peptide (equivalent in 5' → 3' exonuclease activity to 6.9 pmoles polymerase I) ○; 2.8S peptide plus large fragment (8.0 pmoles) ●; 2.8S peptide plus large fragment (8.0 pmoles) plus deoxynucleoside triphosphates, △.

fragment excise dimers as oligonucleotides 4 to 8 residues long (4,5) and we have found dimer-containing octanucleotides in the acid-soluble fraction (data not shown).

#### Discussion

Kelly *et al.* (5) have shown that *E. coli* DNA polymerase I can excise thymine dimers from nicked UV-irradiated DNA and from the UV-irradiated homopolymer pair (dT)<sub>200</sub>·(dA)<sub>4000</sub>. Setlow and Kornberg (4) further demonstrated that the polymerase I small fragment is able to degrade the irradiated homopolymer pair to yield products indistinguishable from that formed by the intact enzyme. We have confirmed these observations using specifically nicked UV-irradiated DNA. However, we have also found that the rate of dimer excision catalyzed by the small fragment is only about one-tenth that observed with the intact enzyme. In contrast, there is no difference in 5' → 3' exonuclease activity when the two are compared with 5'-[<sup>32</sup>P](dT)·(dA) as substrate. Although addition of the polymerase I large fragment to the small fragment does not affect significantly the 5' → 3'

exonuclease activity measured with the homopolymer pair (4), it markedly enhances the rate of dimer excision from incised UV-irradiated DNA. We do not know the reason for this effect; however, one possibility is that binding of the small fragment to a nick at or near a thymine dimer may be weaker than to a nick without the dimer. Addition of the large fragment may then stabilize the binding of the small fragment to termini near the dimer.

The observation that addition of the four deoxynucleoside triphosphates strongly stimulates thymine dimer excision by intact polymerase I, and by the combined large and small fragments, is expected, since 5' → 3' exonuclease activity is markedly and specifically enhanced by concurrent DNA synthesis (9). This finding does, however, reinforce the idea that maximal rates of 5' → 3' exonuclease activity, and hence dimer excision, require the concerted action of both polymerase and exonuclease. Thus the finding of nearly normal levels of 5' → 3' DNA polymerase I-associated exonuclease activity in extracts of pol A mutants (1) may not be sufficient to explain the essentially normal rate of dimer excision observed in vivo (10).

The observation that the 2.8S peptide with 5' → 3' exonuclease activity isolated from amber mutants of polymerase I is very similar, if not identical, to the proteolytically generated small fragment in its ability to interact with the large fragment, provides strong support for the notion that this 5' → 3' exonuclease activity is related to the incomplete peptide generated in E. coli pol A1 as a result of the amber mutant which it bears.

#### Acknowledgments

We thank Janice Chien and Karin Goldmann for excellent technical assistance. This work was supported by grants (CA-12428 to ECF) and (GM-06196 to IRL) from the USPHS, and contract AT(04-3)-326 with the U.S. Atomic Energy Commission (to ECF). E.C.F. is a Fellow of the Andrew W. Mellon Foundation.

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