The excision of AP sites by the 3′–5′ exonuclease activity of the Klenow fragment of *Escherichia coli* DNA polymerase I

Véronique Bailly and Walter G. Verly

Laboratoire de Biochimie, Faculté des Sciences, Université de Liège, Sart Tilman B6, 4000 Liège I, Belgium

Received 1 October 1984

The 3′ AP endonucleases (class I) are said to hydrolyze the phosphodiester bond 3′ to AP sites yielding 3′-OH and 5′-phosphate ends; on the other hand, the resulting 3′ terminal AP site is not removed by the 3′–5′ exonuclease activity of the Klenow fragment [1]. We show that AP sites in DNA are easily removed by the 3′–5′ exonuclease activity of the Klenow fragment and that they are excised as deoxyribose-5-phosphate. It is suggested that the 3′ AP endonucleases are perhaps not the hydrolases they are supposed to be.

DNA repair *AP site* *DNA polymerase I* *Klenow fragment* *3′–5′ Exonuclease* *3′ AP endonuclease*

1. INTRODUCTION

Authors in [1] distinguish two classes of AP endonucleases. Enzymes of class I are said to hydrolyze the phosphodiester bond 3′ to the AP site leaving 3′-OH and 5′-phosphate ends; for the sake of clarity, we shall call them 3′ AP endonucleases. The enzymes of class II, which were the first to be discovered, are 5′ AP endonucleases; they hydrolyze the phosphodiester bond 5′ to the AP site leaving 3′-OH and 5′-phosphate ends, as was shown for *E. coli* endonuclease VI [2].

According to authors in [1,3], the 3′ ends left by the 3′ AP endonucleases are not used easily by *E. coli* DNA polymerase I; the 3′–5′ exonuclease activity of this polymerase removes the 3′ terminal AP site with much difficulty. It is necessary to cut away this terminal AP site with a 5′ AP endonuclease to find the 3′-OH end needed for polymerization. Since the initial publication of [1], several authors have identified 3′ AP endonucleases on the basis that nicking depurinated or depyrimidinated DNA with them does not leave a good substrate for DNA polymerase I [4–6].

Here, we show that a 3′ terminal AP site with a 3′-OH is easily removed by the 3′–5′ exonuclease activity of the Klenow fragment of *E. coli* DNA polymerase I. We conclude that if the 3′ AP endonucleases do indeed nick 3′ to the AP site, they probably do not leave 3′-OH ends.

2. MATERIALS AND METHODS

2.1. *The enzymes*

2.1.1. *E. coli* DNA polymerase I and its large fragment

Endonuclease-free DNA polymerase I from *E. coli* was purchased from Boehringer, as was also the large Klenow fragment. The absence of uracil-DNA glycosylase activity was checked on [^{3}H]uracil-containing DNA; the absence of non-specific endonucleases was checked on phage ϕX174 RF-I DNA and the absence of AP endonuclease activity on this RF-I DNA containing AP sites. The enzyme concentrations were those used in section 3; the results indicate, in particular, that the Klenow fragment preparation did not contain any endonuclease VI/exonuclease III activity.

2.1.2. Uracil-DNA glycosylase

The enzyme was prepared from *E. coli* B41

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

following the method of [7]. The preparation was also checked on phage ϕX174 RF-I DNA, with and without AP sites: the uracil-DNA glycosylase was not contaminated by AP endonuclease or non-specific endonuclease activities.

E. coli exonuclease III was purchased from B.R.L. and pancreatic DNase from Boehringer.

2.2. *The two doubly-labelled DNA substrates*

2.2.1. The uracil-containing doubly-labelled DNA

[α-^{32}P]dCTP (Amersham; 410 Ci/mmol) was deaminated by treatment with NaOH as described [8]. Analysis of the reaction mixture by thin layer chromatography on PEI-cellulose indicated that 42% of the radioactivity was in dUTP, 8% in dCTP and the remaining in nucleoside mono- and di-phosphates. The reaction mixture was used as such for DNA synthesis.

[^{3}H]DNA (55000 dpm/μg) was prepared from *E. coli* grown in the presence of [^{3}H]thymidine. It was treated with pancreatic DNase I to have an acid-soluble fraction of 0.18%.

This nicked [^{3}H]DNA (150 μg) was incubated in 6 ml of 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol (pH 7.5), with 150 nmol dATP, 150 nmol dGTP, 150 nmol dTTP, 30 nmol [α-^{32}P]dUTP (0.71 Ci/mmol), 750 nmol dCTP, and 100 units of endonuclease-free *E. coli* DNA polymerase I. (More dCTP was added to dilute the [α-^{32}P]dCTP brought with the [α-^{32}P]dUTP so that the ratio of dCTP/dUTP specific radioactivities was less than 1%.) After 30 min at 15°C, the mixture was deproteinized with chloroform, and dialyzed 4 times to be finally in 50 mM Hepes–KOH, 1 mM EDTA, 1 mM 2-mercaptoethanol (pH 8.0). The doubly-labelled DNA (25 μg/ml) had specific radioactivities of 52800 dpm ^{3}H and 8100 dpm ^{32}P per μg. Nick translation had replaced 4% of the [^{3}H]DNA by [^{32}P]DNA. The acid-soluble fraction was 0.20%. The localization of the two isotopes was checked by degradation with *E. coli* exonuclease III; as shown in fig.1, the ^{32}P was indeed released in acid-soluble fragments much faster than the ^{3}H.

2.2.2. The AP site-containing doubly-labelled DNA

A part of the doubly-labelled uracil-containing DNA preparation was incubated with the uracil-DNA glycosylase and the progress of the reaction followed by the determination of the acid-soluble radioactivity after an alkaline treatment [9]. The reaction was stopped when this acid-soluble radioactivity reached a plateau. In the absence of an alkaline treatment, the acid-soluble radioactivity of the nicked, doubly-labelled, AP site-containing DNA was always 0.20%.

2.3. *DEAE-Sephadex A25 chromatographies*

2.3.1. In the presence of 7 M urea

The sample, diluted in 5–10 ml of buffer A (5 mM Tris–HCl, 7 M urea, pH 7.6), was placed on top of a 60 × 0.9 cm column of DEAE-Sephadex A25 (Pharmacia) equilibrated with buffer A. The elution was carried out with 700 ml of a linear 0–0.4 M LiCl gradient in buffer A at a rate of 30 ml/h; 4-ml fractions were collected. This chromatography enabled separation of mono-, di-, tri- and tetra-deoxynucleotides. An absorbance marker of dUMP was added to the sample to give the elution position of this deoxynucleoside monophosphate.

2.3.2. In the absence of urea

The sample, diluted in 5 ml of buffer B (0.1 M Tris–HCl, pH 8.3), was placed on top of a 30 × 0.9 cm column of DEAE-Sephadex A25 equilibrated with buffer B. The elution was carried out with 250 ml of a linear 0–0.3 M NaCl gradient in buffer B at a rate of 25 ml/h; 3-ml fractions were collected. This chromatography enabled separation of deoxyribose-5-phosphate and the different deoxynucleoside monophosphates. Non-radioactive deoxyribose-5-phosphate (PL Biochemicals), dUMP and dCMP were added to the sample. The elution position of the deoxyribose-5-phosphate was read at 600 nm after reaction with diphenylamine [10]; those of dUMP and dCMP at 260 nm.

2.4. *Miscellaneous*

2.4.1. Acid-soluble fraction

To 20 μl of the sample were added 100 μl 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0) containing 200 μg calf thymus DNA and 240 μl 7.5% $HClO_4$. After vortexing, the tube was left 10 min on ice, then centrifuged 10 min at 12000 × g and the radioactivity of the supernatant was determined. The result is expressed in percent of the sample total radioactivity.

2.4.2. Norit-non-adsorbable fraction

The acid-soluble fraction (300 μl) was neutralized with 300 μl 1 M KOH; after a few min on ice, the tube was centrifuged 5 min at 12000 × *g*. To 400 μl of the supernatant were added 400 μl 0.25 M Tris–HCl, 25 mM potassium phosphate, 20 mM sodium pyrophosphate (pH 8.5), and 200 μl 50% Norit in water. After 10 min on ice, the tube was centrifuged 15 min at 17500 × *g* and the radioactivity of the supernatant was determined.

3. EXPERIMENTS AND RESULTS

The uracil-containing doubly-labelled DNA was labelled with ^{3}H in thymine moieties except for a few percent at the 3′ ends of the strand pieces which were labelled with ^{32}P specifically 5′ to the uracil residues. In the AP site-containing doubly-labelled DNA, the uracil residues had been removed giving AP sites; the 3′ ends were labelled with ^{32}P specifically 5′ to these AP sites.

One or the other of these two DNA substrates (20 μg) and 50 units of the Klenow fragment of *E. coli* DNA polymerase I in 700 μl of solution (pH 8.0) containing 0.01 M $MgCl_2$, were incubated at 37°C up to 60 min; aliquots were taken at different times to measure the ^{32}P and ^{3}H acid-soluble radioactivities. A control incubated without the Klenow fragment was used to correct the results which were below 0.5%. Fig.2A,B shows that, with both substrates, the ^{32}P was released in the acid-soluble fraction much faster than the ^{3}H, exactly as when *E. coli* exonuclease III was used to degrade the uracil-containing doubly-labelled DNA (fig.1). The conclusion is that an AP site is not a significant obstacle to the 3′–5′ exonuclease activity of the Klenow fragment. The rate of ^{32}P appearance in the acid-soluble fraction, however, was slower when there were AP sites: after 15 min, 50% of the ^{32}P was in the acid-soluble fraction when the uracil-containing substrate was used, against 19% for the AP site-containing substrate.

The acid-soluble fractions of aliquots taken after 60-min incubation with the Klenow fragment were tested for Norit-non-adsorbable ^{32}P. With the uracil-containing substrate, the non-adsorbable ^{32}P was only 1%, whereas with the AP site-containing substrate, it amounted to 92%.

At the end of the 60-min incubation, the reactions were stopped by addition of 150 μl 0.1 M EDTA to the remaining solution; 300 μl were then used for DEAE-Sephadex A25 chromatography in the presence of urea (fig.3) and 200 μl for DEAE-Sephadex A25 chromatography in absence of urea (fig.4).

When the uracil-containing substrate was used, the ^{32}P radioactivity peak was coincident with the

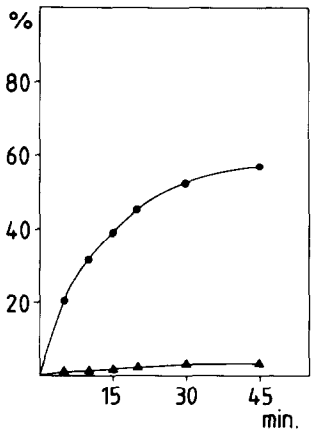

Fig.1. Degradation of uracil-containing doubly-labelled DNA by *E. coli* exonuclease III. After different incubation times (min), the ^{32}P (●) and ^{3}H (▲) acid-soluble radioactivities were measured; the results are expressed as % of the total radioactivities.

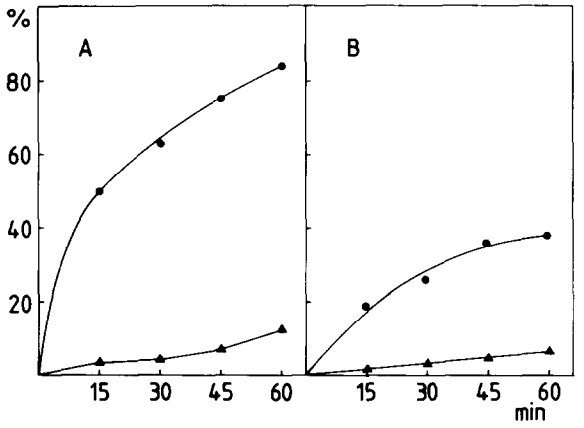

Fig.2. Degradation of the doubly-labelled DNAs by the Klenow fragment of *E. coli* DNA polymerase I. The uracil-containing (A) or the AP site-containing (B) doubly-labelled DNA was incubated with the Klenow fragment of *E. coli* DNA polymerase I. After different times (min), the ^{32}P (●) and ^{3}H (▲) acid-soluble radioactivities were measured; the results are expressed as % of the total radioactivities.

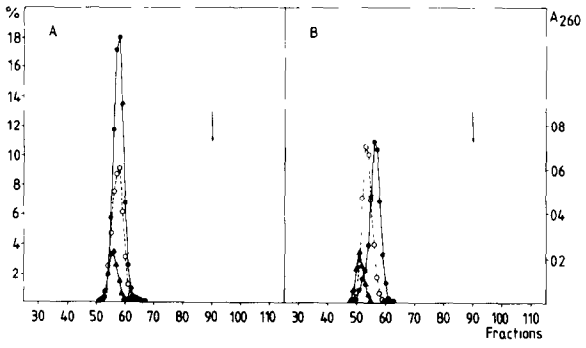

Fig.3. DEAE-Sephadex chromatography in presence of urea. After a 60-min incubation, with the Klenow fragment of *E. coli* DNA polymerase I, of the uracil-containing (A) or the AP site-containing (B) doubly-labelled DNA, dUMP was added as marker and the mixture was chromatographed on DEAE-Sephadex A25 in presence of urea. The absorbance at 260 nm (○), the ^{32}P (●) and ^{3}H (▲) radioactivities were measured on each fraction; the latter results are expressed as % of the total radioactivities. No radioactivity was eluted after fraction 65. The arrow indicates the elution position of dinucleotides.

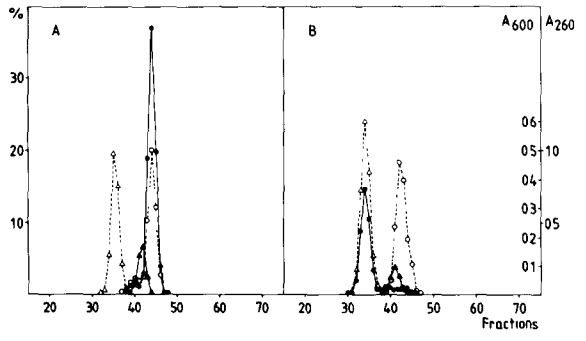

Fig.4. DEAE-Sephadex chromatography in absence of urea. After a 60-min incubation, with the Klenow fragment of *E. coli* DNA polymerase I, of the uracil-containing (A) or the AP site-containing (B) doubly-labelled DNA, dUMP and deoxyribose-5-phosphate were added as markers and the mixture was chromatographed on DEAE-Sephadex A25 in the absence of urea. The absorbance at 260 nm (○) or, after reaction with diphenylamine, at 600 nm (△), the ^{32}P (●) and ^{3}H (▲) radioactivities were measured on each fraction; the latter results are expressed as % of the total radioactivities. No radioactivity was eluted after fraction 50.

peak of the dUMP marker whatever the chromatography, in the presence (fig.3A) or absence (fig.4A) of urea.

When the AP site-containing substrate was used and the DEAE-Sephadex A25 chromatography carried out in the presence of urea, the ^{32}P and ^{3}H peaks were found in the region of the monodeoxynucleotides (fig.3B). When the chromatography was carried out in the absence of urea to clearly separate the deoxyribose-5-phosphate from the monodeoxynucleotides, the ^{3}H peak was near the peak of the dUMP marker (the dCTP marker is not eluted from the column) whereas the ^{32}P peak was exactly coincident with the peak of the deoxyribose-5-phosphate marker (fig.4B). The conclusion is that the AP site is removed by the 3′–5′ exonuclease activity of the Klenow fragment as deoxyribose-5-phosphate.

4. DISCUSSION

Authors in [3] observed that, after nicking 3′ to an AP site with *E. coli* endonuclease III (a 3′ AP endonuclease), *E. coli* DNA polymerase I excised only very slowly the AP site and its large Klenow fragment did not excise it at all.

Here, we show that an AP site in DNA is not a serious obstacle to the 3′–5′ exonuclease activity of the Klenow fragment. Moreover, since, in our experiment, the Klenow fragment released the AP site in a deoxyribose-5-phosphate molecule, the enzyme, after removing the preceding monodeoxynucleotide, was in front of the AP site exactly as the authors in [3] supposed that the enzyme was in their experiment.

There is no doubt that, in our experiment, after removing the preceding monodeoxynucleotide, the deoxyribose of the AP site had a free 3′-OH. Such a 3′-OH end seems to be a necessary condition for the 3′–5′ exonuclease activity of DNA polymerase I: a 3′-phosphate or a 3′-phosphoglycolate end prevents the strand degradation [11,12]. The question is whether a 3′-OH end is sufficient for the error-correcting mechanism of DNA polymerase I to take place. Our results with the Klenow fragment suggest an affirmative answer even when no base is attached to the 3′ terminal deoxyribose.

Authors in [5] have shown that the strand nicking 3′ to the AP site by *E. coli* endonuclease III, leaves a 5′-phosphate end; they inferred, from this

result, that the other end was a 3′-OH. Our results rather suggest that nicking by endonuclease III does not leave a 3′-OH end. If this conclusion is correct, the enzyme action on the phosphodiester bond is not the action of a hydrolase and *E. coli* endonuclease III should not be called an AP endonuclease; it ought to altogether change its name because it would not even be an endonuclease. If this remark is applicable to all the 3′ AP endonucleases which give a nicked DNA that cannot support polymerization by *E. coli* DNA polymerase I in spite of the editing function of its 3′–5′ exonuclease activity, it is possible that, after all, there are no such things as class I AP endonucleases. A direct determination of the nature of the 3′ ends of the nicks produced by the so-called 3′ (or class I) AP endonucleases is thus highly needed.

ACKNOWLEDGEMENTS

This work was supported by grants from the Fonds Cancérologique de la C.G.E.R. and the Fonds de la Recherche Scientifique Médicale. V.B. is the recipient of a fellowship from I.R.S.I.A.

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