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Importance of thiols in the repair mechanisms of DNA containing AP (apurinic or apyrimidinic) sites

Véronique Bailly and Walter G.Verly

Department of Biochemistry, Faculty of Sciences, University of Liège, Sart Tilman B6, 4000 Liege 1, Belgium

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ABSTRACT

Addition of thiol compounds containing an anionic group to the 3'-terminal unsaturated sugar of the 5' fragment obtained from an oligonucleotide containing an AP site cleaved by β-elimination, can be followed by gel electrophoresis. The technique enables to distinguish between two mechanisms of cleavage of the $C_{3'}$-O-P bond 3' to an AP site : hydrolysis or β-elimination.

Addition of thiols to the double-bond of the 3'-terminal sugar resulting from β-elimination prevents a subsequent δ-elimination. The interpretation of the action of enzymes that start by nicking 3' to AP sites must take into account the presence or absence of thiols in the reaction medium. In living cells, thiols might influence the pathways followed by the repair processes of AP site-containing DNA.

INTRODUCTION

All the cells seem to possess an AP endonuclease activity that hydrolyzes the phosphodiester bond 5' to AP (apurinic/apyrimidinic) sites in DNA leaving 3'-OH and 5'-phosphate ends [1,2,3,4]. But the excision of an AP site requires another cleavage 3' to the damage. We have shown that the so-called AP endonucleases, class I [5], that break the phosphodiester bond 3' to AP sites leaving 5'-phosphate ends, are not endonucleases, but very likely β-elimination catalysts [6,7]. On the other hand, no mammalian enzyme has been convincingly shown to hydrolyze a phosphodiester bond 3' to an AP site. Bailly and Verly [8] have thus made the hypothesis that, in mammalian cells, the nicking 3' to AP sites is always the result of a β-elimination reaction; histones and polyamines most probably play a major role in the repair of AP sites in these cells.

β-Elimination acting on an AP site in DNA leaves a 3'-terminal α,β-unsaturated aldehyde derived from the base-free deoxyribose. Manoharan et al. [9] have found that thiols react with this unsaturated aldehyde. In this paper, we have investigated possible uses of thiols in the study of AP site repair; we have also stressed a possible influence of thiols on the repair mechanisms.

MATERIALS

The oligonucleotides [^{32}P]pdT$_8$d(-), [^{32}P]pdT$_8$d(-)dT$_7$ and [^{32}P]pdT$_{11}$d(-)dT$_{11}$ dT$_8$dA (Pharmacia), dT$_8$dGdT$_7$ or dT$_{11}$dGdT$_{11}$ (Eurogentec) (10 nmol), [γ-^{32}P]ATP (Amersham; 15 nmol; 150 µCi) and T4 polynucleotide kinase (Pharmacia; 10 units) in 100 µl 60 mM Tris.HCl, pH 8.0, 9 mM $MgCl_2$, 0.1 M KCl, 15 mM 2-mercaptoethanol, were incubated 1 h at 37°C. The reaction was stopped with EDTA. The radiolabelled oligonucleotides were purified on NENSORB cartridges (NEN Research Products). [^{32}P]pdT$_8$dGdT$_7$ and [^{32}P]pdT$_{11}$dGdT$_{11}$ were depurinated by a 24-h incubation in 30 mM HCl at 37°C. [^{32}P]pdT$_8$dA was depurinated by a 1-h incubation at 65°C in 10 mM HCl. The AP site in the depurinated oligonucleotides is noted by d(-).

The enzymes :

E.coli endonuclease III was a gift of Dr. J. Laval (Paris). The preparation was diluted in 50 mM Tris.HCl, pH 8.0, 1 mM EDTA, 50 % glycerol.

T4 UV endonuclease was prepared from an overproducing strain, E.coli AB2480 - ptac denV, constructed by Valerie and de Riel (Temple University, Philadelphia, USA). The enzyme was practically pure after two chromatographies on ssDNA-agarose and CM Sephadex C25. The enzyme was kept at 4°C in 10 mM potassium phosphate, pH 6.5, 10 mM EDTA, 0.5 M KCl, 10 % ethylene glycol. The enzyme was diluted just before utilization in 50 mM Hepes.KOH, pH 7.4, 1 mM EDTA, 50 mM NaCl, 10 % ethylene glycol.

M.luteus UV endonuclease was a gift from Dr. M. Liuzzi (Edmonton, Canada). It was purified according to Paterson et al. [10]. The enzyme was kept at -20°C in 250 mM potassium phosphate, pH 7.5, 2 mM 2-mercaptoethanol, 30 % glycerol.

Miscellaneous :

2-Mercaptoethanol, sodium thioglycolate, sodium 2-mercaptoethanesulfonate, 3-mercaptopropionic acid were bought from Sigma. Polyacrylamide gel electrophoresis and autoradiography were as described by Bailly and Verly [7].

RESULTS

1 - Thiols interfere with the δ-elimination that follows nicking 3' to an AP site by β-elimination. Thiols enable to distinguish between a 3'-terminal base-free deoxyribose and a 3'-terminal unsaturated sugar derivative resulting from the nicking 3' to an AP site by β-elimination :

[^{32}P]pdT$_8$d(-)dT$_7$ nicked with spermine and [^{32}P]pdT$_8$d(-) with a 3'-terminal base-free deoxyribose were compared. The reaction products were submitted to electrophoresis on a polyacrylamide gel which was subsequently autoradiographed (figure 1).

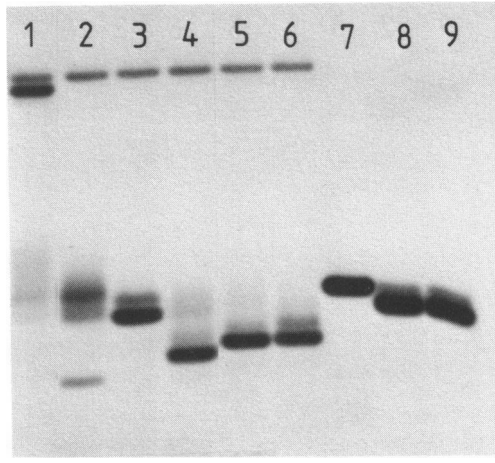

Figure 1 : Reaction with thiols of the unsaturated sugar produced by nicking the DNA strand 3' to an AP site by β-elimination; suppression of δ-elimination.

[32P]pdT8d(-)dT7 (lane 1) nicked with spermine in absence (lane 2) or in the presence of 10 mM 2-mercaptoethanol (lane 3), thioglycolate (lane 4), 2-mercaptoethanesulfonate (lane 5) or 3-mercaptopropionate (lane 6). [32P]pdT8dA (lane 7); [32P]pdT8d(-) incubated in absence (lane 8) or in the presence of 10 mM thioglycolate (lane 9).

- Lane 1 : $[^{32}P]pdT_8d(-)dT_7$. The depurination was not complete so that, besides the major band of $[^{32}P]pdT_8d(-)dT_7$, persists a minor one of $[^{32}P]pdT_8dGdT_7$;
- Lane 2 : $[^{32}P]pdT_8d(-)dT_7$ (320 pmol nucleotides), hybridized to polydA (640 pmol nucleotides), in 10 µl 50 mM Hepes.KOH, pH 8.0, 1 mM EDTA, 1 mM spermine, was incubated 1 h at 37°C. The residual $[^{32}P]pdT_8dGdT_7$ band was unchanged, but the $[^{32}P]pdT_8d(-)dT_7$ has completely disappeared; it is replaced by compounds forming three bands : a doublet that we represent by $[^{32}P]pdT_8s(=)$ resulting from the β-elimination and $[^{32}P]pdT_8p$ resulting from βδ-elimination (see also Bailly and Verly, [7]);
- Lane 3 : Same experiment as described for lane 2, except that the medium also contained 10 mM 2-mercaptoethanol. There is only a slight displacement of the doublet, but the δ-elimination has been completely prevented;
- Lanes 4, 5 and 6 : To have a significant displacement of the β-elimination products after addition of the thiol on the α,β double bond of the unsaturated sugar derivative, the 2-mercaptoethanol was replaced by a thiol compound with an anionic group. The treatments were the same as described for lane 2, except that

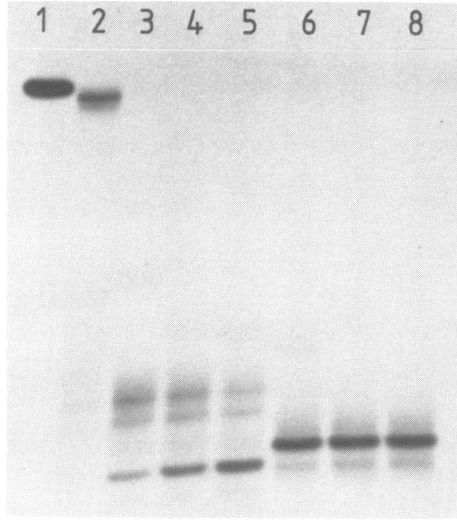

Figure 2 : Reaction of the β-elimination products with thioglycolate interferes with δ-elimination.

[32P]pdT11dGdT11 (lane 1) and [32P]pdT11d(-)dT11 (lane 2). Incubation of [32P]pdT11d(-)dT11 with spermine for 1 h (lanes 3 and 6), 3 h (lanes 4 and 7) or 6 h (lanes 5 and 8), in absence (lanes 3, 4 and 5) or presence (lanes 6, 7 and 8) of thioglycolate.

the incubation medium contained 10 mM thioglycolate (lane 4), 2-mercaptoethanesulfonate (lane 5) or 3-mercaptopropionate (lane 6). One can see, especially clearly in lanes 5 and 6, that the primary products of β-elimination which formed the doublet [^{32}P]pdT$_8$s(=) have been replaced by secondary products that migrated significantly farther;

- Lanes 7 and 8 : [^{32}P]pdT$_8$dA or [^{32}P]pdT$_8$d(-) respectively (180 pmol nucleotides), with polydA (640 pmol nucleotides), in 10 µl 50 mM Hepes.KOH, pH 8.0, 1 mM EDTA, was incubated 1 h at 37°C. Lane 8 shows that the [^{32}P]pdT$_8$d(-) preparation still contained some not-depurinated [^{32}P]pdT$_8$dA;

- Lane 9 : Same experiment as described for lane 8 except that the medium contained 10 mM thioglycolate. The result is exactly the same as without thioglycolate : the thiol did not react with the 3'-terminal base-free deoxyribose.

To better show the effect of thiols on the δ-elimination, in figure 2, treatments of increasing durations (lanes 3 and 6 : 1 h; lanes 4 and 7 : 3 h; lanes 5 and 8 : 6 h) of [^{32}P]pdT$_{11}$d(-)dT$_{11}$, hybridized to polydA, with 1 mM spermine were carried out in absence (lanes 3, 4 and 5) or in the presence (lanes 6, 7 and 8) of 10 mM

thioglycolate (the other experimental details are the same as for figure 1, lanes 2 and 3). In this experiment, $[^{32}P]pdT_{11}dGdT_{11}$ (lane 1) was completely depurinated into $[^{32}P]pdT_{11}d(-)dT_{11}$ (lane 2). The shortest incubation time with spermine was sufficient to degrade completely $[^{32}P]pdT_{11}d(-)dT_{11}$ (lane 3), but, when there was no thioglycolate, with increasing time, the importance of the β-elimination doublet $[^{32}P]pdT_{11}s(=)$ decreased whereas that of $[^{32}P]pdT_{11}p$, produced by a subsequent δ-elimination, increased (compare lanes 3, 4 and 5). In the presence of thioglycolate, the β-elimination and the βδ-elimination bands essentially vanished to be replaced by a band resulting from the addition of the thiol to the β-elimination products (lanes 6, 7 and 8).

2 - The use of thioglycolate to identify the nature of the nicking 3' to an AP site by E.coli endonuclease III, T4 or M.luteus UV endonuclease :

The longer oligonucleotide $[^{32}P]pdT_{11}d(-)dT_{11}$, hybridized to polydA, was used. After various treatments, the products were analyzed as before (figure 3).

- Lanes 3 and 4 : $[^{32}P]pdT_{11}d(-)dT_{11}$ submitted to a mild alkaline treatment (15 min at 37°C in 0.2 M NaOH; lane 3) or to a strong alkaline treatment (30 min at 65°C in 0.2 M NaOH; lane 4). The mild treatment nicked all the molecules 3' to theAP site by β-elimination yielding the doublet $[^{32}P]pdT_{11}s(=)$; quite often, the β-elimination was followed by a δ-elimination giving $[^{32}P]pdT_{11}p$. During the strong alkaline treatment, all the molecules have undergone a βδ-elimination reaction yielding $[^{32}P]pdT_{11}p$ as the only significant product.

- Lanes 5 and 6 : $[^{32}P]pdT_{11}d(-)dT_{11}$ (460 pmol nucleotides), hybridized to polydA (920 pmol nucleotides), and E.coli endonuclease III in 10 µl 50 mM Hepes.KOH, pH 7.5, 0.1 M KCl, 1 mM EDTA, without (lane 5) or with 10 mM thioglycolate (lane 6), were incubated 1 h at 37°C. In the thiol-free medium, the enzyme gave the doublet characteristic of β-elimination (compare with lane 3). When thioglycolate was present, a small part of the β-elimination products has reacted with the thiol to give two adducts that migrated farther.

- Lanes 7, 8, 9 and 10 : The same amount of substrate, hybridized to polydA, and T4 (lanes 7 and 8) or M.luteus (lanes 9 and 10) UV endonuclease in 10 µl 50 mM Hepes.KOH, pH 7.4, 50 mM NaCl, 1 mM EDTA, without (lanes 7 and 9) or with 10 mM thioglycolate (lanes 8 and 10) were incubated 1 h at 37°C. Like E.coli endonuclease III, the two UV endonucleases gave the β-elimination doublet in the medium devoid of thioglycolate (lanes 7 and 9). When T4 UV endonuclease acted in the presence of thioglycolate (lane 8), the two β-elimination products almost disappeared to be replaced by a lower band resulting from the addition of thioglycolate. The same band, less important however, is also observed when the nicking was performed by M.luteus UV endonuclease in the presence of thio-

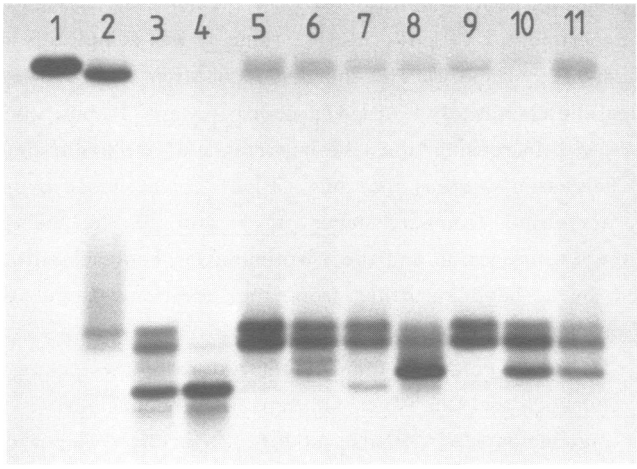

Figure 3 : Study, with thioglycolate, of the nicking mechanism 3' to AP sites by E.coli endonuclease III, T4 UV endonuclease, and M.luteus UV endonuclease.

[32P]pdT11dGdT11 (lane 1) and [32P]pdT11d(-)dT11 (lane 2). [32P]pdT11d(-)dT11 submitted to mild alkaline treatment (lane 3) or strong alkaline treatment (lane 4). [32P]pdT11d(-)dT11, hybridized to polydA, nicked with E.coli endonuclease III (lanes 5 and 6), T4 UV endonuclease (lanes 7 and 8) or M.luteus UV endonuclease (lanes 9 and 10) in absence (lanes 5, 7 and 9) or in the presence of 10 mM thioglycolate (lanes 6, 8 and 10).

glycolate (lane 10); the remaining doublet, with the lower band more intense than the upper one, is probably explained by the presence of β-mercaptoethanol in the M.luteus UV endonuclease preparation : addition of β-mercaptoethanol instead of thioglycolate on the β-elimination products likely gives the reason for the differences observed between lane 10 (M.luteus UV endonuclease) and lane 8 (T4 UV endonuclease).

DISCUSSION

Nicking 3' to an AP site in DNA by β-elimination leaves a 2',3'-unsaturated sugar at the 3' end of the 5' fragment. Manoharan et al. [9] observed that, in the presence of nucleophiles such as thiols, the β-elimination primary product was transformed by addition on the double-bond.

We previously reported that nicking 3' to AP sites by β-elimination yields two different 5' fragments forming a doublet on the electrophoretogram [7]. Manoharan et al. [9] have speculated that addition of a thiol explained our observation. We do not think that it is the right interpretation since the doublet is observed after a mild alkaline treatment in a medium that does not contain any thiol (see, for

instance, figure 3, lane 3). Moreover the disappearance of the β-elimination doublet when a thiol is in the incubation medium as we shall see later, indicates that the formation of the β-elimination doublet does not necessitate addition of a nucleophile on the double-bond. We do not know why there are always two 5' fragments; one may think of a cis-trans isomerism, or of a spontaneous conversion of the primary product into another one bearing, for instance, a 2-oxocyclopent--1-enyl [11] at its 3' end. Whatever the explanation, the doublet observed on the electrophoretogram is the signature of nicking by β-elimination. Indeed, when the 5'-labelled oligonucleotide containing an AP site is degraded by snake venom phosphodiesterase, the last phosphoester bond hydrolyzed by this 3'-5' exonuclease is the $C_{3'}$-O-P immediately on the 3' side of the AP site and the shortened oligonucleotide with a base-free deoxyribose at the 3' end gives a single band after polyacrylamide gel electrophoresis and autoradiography (data not shown).

Using $[^{32}P]pdT_8d(-)dT_7$ or $[^{32}P]pdT_{11}d(-)dT_{11}$ and the technique of gel electrophoresis, we show, in this paper, that the 5' products of β-elimination react with thiols as suggested by Manoharan et al. [9]; the nicking was obtained with spermine which gave the classical doublet plus a band of βδ-elimination (figure 1, lane 2; figure 2, lanes 3, 4 and 5; see also Bailly and Verly, [7,8]). The demonstration could not be done with 2-mercaptoethanol because the position of the adducts was not very different from that of the primary products of β-elimination. But the addition reaction was clearly seen when we used molecules having an anionic group in addition to the thiol group, such as thioglycolate, 2-mercaptoethanesulfonate or 3-mercaptopropionate (see figure 1, lanes 4, 5 and 6 to be compared with lane 2). We also show that no such addition occurred when the 3'-terminal base-free sugar was deoxyribose (figure 1, lane 9 to be compared with lane 8) as one should expect. The conclusion is that treatment with a thiol containing an anionic group can be used to investigate the nature of the 3' end of the 5' fragment left by a nicking enzyme acting 3' to an AP site in order to draw a conclusion on the nicking mechanism : hydrolysis that leaves a deoxyribose or β-elimination that leaves an unsaturated sugar. In figure 3, we have applied the technique to three enzymes (E.coli endonuclease III, T4 and M.luteus UV endonucleases) which have been erroneously classified as endonucleases and lately as AP endonucleases, class I [5]. In absence of thiol, the three enzymes, acting on $[^{32}P]pdT_{11}d(-)dT_{11}$ hybridized to polydA, gave the doublet characteristic of β-elimination; moreover, in the presence of thioglycolate, part of the primary products reacted with the thiol to give compounds that migrated farther on the electrophoretogram (figure 3, lanes 6, 8 and 10).

We have also noticed that, when the β-elimination occurred in the presence of

thiols, the δ-elimination was prevented (figure 1, compare lanes 2 and 3; figure 2, compare lanes 3, 4 and 5 to lanes 6, 7 and 8). The 3' nicking by β-elimination of some enzymes acting on AP sites is sometimes followed by a 5' nicking by δ-elimination (unpublished results), but it is now obvious that the presence or absence of thiol in the incubation medium can change the issue. We have, for instance, published that E.coli endonuclease III nicks 3' to AP sites by catalyzing a β-elimination [7]; the β-elimination was not followed by any δ-elimination, but the incubation medium contained 2-mercaptoethanol and this thiol might have been the cause of the absence of δ-elimination. In this paper, we have repeated a similar experiment; figure 3, lane 5, shows that, even in absence of thiol, the β-elimination was not followed by δ-elimination. For all these enzymes, the most interesting situation is obviously that existing in the cell where thiols are present; these thiols might have an influence on the pathways followed to repair DNA containing AP sites that deserves to be studied.

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