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The relative importance of *Escherichia coli* exonuclease III and endonuclease IV for the hydrolysis of 3'-phosphoglycolate ends in polydeoxynucleotides

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ABSTRACT

In vitro, in the presence of Mg^{++}, the 3'-phosphoglycolatase activity of endonuclease IV is about 4-times smaller than that of exonuclease III for the same AP endonuclease activity. It thus seems that endonuclease IV has only a minor role in the repair of strand breaks limited by 3'-phosphoglycolate ends in Escherichia coli even after the amount of enzyme has been increased by induction with O_2^--generating agents.

INTRODUCTION

Endonuclease VI [1,2] and endonuclease IV [3] are the two major AP (apurinic/apyrimidinic) endonucleases of E.coli. Endonuclease VI counts for 90 % and endonuclease IV for 10 % of the bacteria AP endonuclease activity [4]. Endonuclease VI, encoded in the xth gene, is the same protein as exonuclease III [4,5,2].

Strand breaks in DNA due to ionizing radiations are limited, on one side, by 5'-phosphate ends and, on the other side, by 3'-phosphate or 3'-phosphoglycolate ends [6]. Exonuclease III hydrolyzes 3'-phosphate and 3'-phosphoglycolate into 3'-hydroxyl [7]. Mutants xth^- are not hypersensitive to ionizing radiations [8] although they repair more slowly X-ray-induced strand breaks [9]. But endonuclease IV also hydrolyzes 3'-phosphoglycolate ends into 3'-hydroxyl [10]. On the other hand, agents generating O_2^- and capable to produce 3'-phosphoglycolate ends increase the amount of endonuclease IV in E.coli; the induction can heighten the amount of enzyme so that, in the induced bacteria, the AP endonuclease activities of endonuclease VI-exonuclease III and of endonuclease IV are about equal [11]. In this work, we try to estimate the relative importance of the 3'- phosphoglycolatase activities of exonuclease III and endonuclease IV in-induced and non-induced E.coli cells.

MATERIALS and METHODS

The enzymes :

Exonuclease III was bought from BRL. Endonuclease IV was prepared by the method of Ljungquist [3] from E.coli B41 induced by paraquat [11].

Synthesis of pdT_{20}-[^{32}P]phosphoglycolate :

A mixture of 100 µl pdT_{20} (19 nmol), 8.8 µl 230 µM [α-^{32}P]dATP (2.0 nmol; 50 µCi), 1 µl terminal deoxynucleotidyl transferase solution (14 units; BRL), and 50 µl 500 mM K cacodylate, pH 7.2, 10 mM $CoCl_2$, 1 mM dithiothreitol, was incubated 4 h at 37°C. The reaction was stopped with 10 µl 50 mM EDTA.

The pdT_{20}[^{32}P]dA was isolated from the reaction mixture by HPLC on a reverse phase column Bondapak C18 (Waters) equilibrated with 0.1 M triethylamine, adjusted at pH 7.0 with acetic acid, containing 10 % acetonitrile; the elution was carried out with a 10 to 20 % acetonitrile linear gradient in the same triethylamine acetate solution in 30 min at a rate of 1 ml/min; 5 µl from each 1-ml fraction were used for radioactivity measurement and the two fractions of the maximum of the radioactive peak were mixed and lyophilized.

pdT_{20}[^{32}P]dA was dissolved in 100 µl 10 mM HCl and depurinated by incubation at 65°C for 1 h to yield pdT_{20}[^{32}P]d(-) [where d(-) indicates the 3'-terminal apurinic site]; the solution was neutralized with 2.5 µl 0.4 M NaOH.

After addition of 15 µl 0.1 M Na borate, pH 9.8, and 30 µl 2 M $NaBH_4$ in 0.05 M NaOH, pdT_{20}[^{32}P]d(-) was reduced at room temperature to open the sugar ring; after 30 min, the reaction was stopped with 15 µl 1 M Na acetate/acetic acid, pH 3.5, and the solution left for 15 h at 4°C.

A freshly prepared 0.3 M $NaIO_4$ solution (30 µl) was added and the mixture kept in the dark at 0°C for 2 h to cleave the 3',4'-diol of the reduced deoxyribose. The pdT_{20}-[^{32}P]phosphoglycolaldehyde so obtained was oxidized into pdT_{20}-[^{32}P]phosphoglycolate in the following way. The solution pH was brought to 8 with 75 µl 1 M Na_2CO_3; after addition of 25 µl 40 mM I_2 (Lugol), it was left 3 h at room temperature, then acidified with 75 µl 1 M HCl; I_2 excess was destroyed with 60 µl 75 mM $Na_2S_2O_3$.

The successive steps of the preparation were followed by gel electrophoresis. The final product was adsorbed on a small NENSORB column and eluted with 20 % ethanol. After evaporation to dryness, the residue was dissolved in 20 µl stop solution (90 % formamide in 0.1 M Tris.borate, pH 8.3, 2 mM EDTA, 0.02 % bromophenol blue, 0.02 % xylene cyanol) and submitted to electrophoresis on a 20 % polyacrylamide gel. The pdT_{20}-[^{32}P]phosphoglycolate was eluted with 1 ml 0.1 M Tris.HCl, 1 mM EDTA, pH 7.5, and desalted on a small NENSORB column.

To show that it did indeed contain a 3'-[^{32}P]phosphoglycolate end,

pdT_{20}-[^{32}P]phosphoglycolate was treated with exonuclease III and the products of the reaction analyzed by HPLC [6]. A mixture of 5 µl pdT_{20}-[^{32}P]phosphoglycolate solution (11 pmol nucleotide), 1 µl pdA_{40-60} solution (300 pmol nucleotide), 5 µl 300 mM Tris.HCl, 160 mM $MgCl_2$, 160 mM NaCl, pH 7.5, 1 µl exonuclease III solution (65 units) and 38 µl H_2O, was incubated for 30 min at 37°C. After addition of 100 µl phosphoglycolic acid (1 mg) solution, 100 µl were injected in an Altech-NH_2 column (250 x 4.6 mm) equilibrated with 0.3 M K phosphate, pH 4.5. The elution was carried out with the same phosphate solution at a rate of 1 ml/min. The absorbance was monitored at 220 nm to locate the phosphoglycolate peak. Fractions of 1 ml were collected and analyzed for radioactivity : 86 % of the injected radioactivity were recovered in the phosphoglycolate peak.

Synthesis of [5'-^{32}P]pdT_8-phosphoglycolate :

A mixture of 120 µl 67 µM dT_8dA (8 nmol; Pharmacia), 30 µl 1.2mM [γ-^{32}P]ATP (37 nmol; 50 µCi), 3 µl T4 polynucleotide kinase solution (30 units; BRL), 15 µl 600 mM Tris.HCl, pH 8.0, 90 mM $MgCl_2$, 150 mM 2-mercaptoethanol, 1 M KCl, was incubated for 1 h at 37°C. The reaction was stopped with 20 µl 0.2 M EDTA. The labelled oligonucleotide was purified by NENSORB chromatography, eluted in 20 % ethanol and dried.

The [5'-^{32}P]pdT_8dA was then submitted to depurination, reduction, oxidation with $NaIO_4$, oxidation with I_2, as described above; all steps were controlled by gel electrophoresis. The final product was desalted on NENSORB and the [5'-^{32}P]pdT_8--phosphoglycolate purified as described above for the pdT_{20}-[^{32}P]phosphoglycolate.

Gel electrophoresis and autoradiography :

Denaturing gels (20 % polyacrylamide) were prepared from 60 g urea, 60 ml 38 % acrylamide/2 % bisacrylamide, 12 ml Tris.borate, pH 8.3, 20 mM EDTA, 4 ml water, 800 µl 10 % ammonium persulfate and 36 µl tetramethylethylenediamine. The 33 x 40 cm gels had a 0.8 mm thickness. The oligonucleotides to be analyzed were dried, dissolved in stop solution, and 5-10 µl were placed in the wells (12 x 0.8 mm).

When the electrophoresis was performed for preparative purpose, a Fuji X-ray film was applied on the gel for a few minutes and, after development of the film, the region of the gel corresponding to the chosen area was cut out with a scalpel and extracted. The analytical gels were exposed for a few hours at -40°C.

Preparation of [5'-^{32}P]$pdT_8d(-)dT_7$:

dT_8dGdT_7 (10 nmol; Eurogentec), [γ-^{32}P]ATP (50 nmol, 120 µCi; Amersham) and T4 polynucleotide kinase (10 units; Pharmacia) in 70 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 20 µl 0.2 M EDTA. The 5'-labelled oligonucleotide was

purified by NENSORB chromatography, recovered in 20 % ethanol and dried.

[5'-^{32}P]pdT$_8$dGdT$_7$ (3 nmol) in 100 µl 30 mM HCl was incubated 24 h at 37°C; the solution of depurinated product [5'-^{32}P]pdT$_8$d(-)dT$_7$ was then neutralized with 7.5 µl 0.4 M NaOH.

EXPERIMENTS and RESULTS

Comparison of the actions of exonuclease III and endonuclease IV on a 5'-labelled substrate with a 3'-phosphoglycolate end.

A mixture of 5 µl [5'-^{32}P]pdT$_8$-phosphoglycolate (0.7 pmol nucleotide) solution, 1 µl pdA$_{40-60}$ (300 pmol nucleotide) solution, 10 µl buffer, amount of exonuclease III or endonuclease IV having approximately the same AP endonuclease activity, and water to have a total volume of 100 µl, was incubated for 30 min at 16°C. The buffer was 300 mM Tris.HCl, pH 7.5, 160 mM $MgCl_2$, 160 mM NaCl, for exonuclease III; it was 500 mM Hepes.KOH, pH 8.2, 2 M NaCl, 10 mM EDTA, 10 mM dithiothreitol, 0.5 % bovine serum albumin, for endonuclease IV.

At the end of the incubation, the samples were deproteinized with chloroform:isoamyl alcohol (24:1; v:v), then analyzed by electrophoresis on 20% polyacrylamide gel that was autoradiographed. Figure 1, lane 1, shows that exonuclease III seems not to have left any substrate; a band of [5'-^{32}P]pdT$_8$-OH results from the release of phosphoglycolate; additional bands of [5'-^{32}P]pdT$_7$-OH, [5'-^{32}P]pdT$_6$-OH and [5'-^{32}P]pdT$_5$-OH, were the result of the 3'-5' exonuclease activity of exonuclease III (the limited degradation is likely due to the melting of

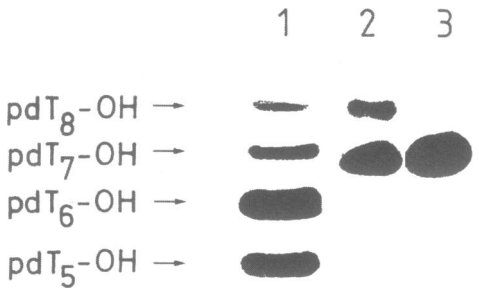

Figure 1 : Action of exonuclease III and endonuclease IV on [5'-^{32}P]-pdT$_8$--phosphoglycolate.

[5'-^{32}P]pdT$_8$-phosphoglycolate (0.7 pmol nucleotide), hybridized to pdA$_{40-60}$, was incubated, for 30 min at 16°C, with amounts of exonuclease III (lane 1) or endonuclease IV (lane 2) having about the same AP endonuclease activity. After deproteinization, the reaction products were analyzed by electrophoresis on polyacrylamide gel that was subsequently autoradiographed. Lane 3 = [5'-^{32}P]-pdT$_8$-phosphoglycolate reference standard.

the double-stranded substrate). Figure 1, lane 2, shows that endonuclease IV has hydrolyzed into [5'-^{32}P]pdT$_8$-OH only a part of the substrate and that the release of the phosphoglycolate was not followed by an exonucleolytic degradation.

Quantitative comparison of the AP endonuclease and 3'-phosphoglycolatase activities of exonuclease III and endonuclease IV.

Since our objective is to try to estimate the relative importance of the two enzymes in the repair of nicks limited by 3'-phosphoglycolate ends in the living bacteria, the experiments have been performed at 37°C and with the same buffer containing Mg^{++}. We shall determine the amounts of the two enzymes having the same AP endonuclease activity on the one hand, and the same 3'-phosphoglycolatase activity on the other hand; the two sets of results will then be compared. Since we are interested only in relative values, the amount of enzyme in 10 µl of the stock solution was set arbitrarily at 100,000 units for exonuclease III as well as for endonuclease IV.

The AP endonuclease activity was measured in the following way. A mixture of 40 µl [5'-^{32}P]pdT$_8$d(-)dT$_7$ (1536 pmol nucleotide) and polydA (4700 pmol nucleotide) in twice-diluted Hepes buffer (100 mM Hepes.KOH, 20 mM $MgCl_2$, 100 mM NaCl, 2 mM dithiothreitol, pH 8.0) and 10 µl of diluted enzyme solution was incubated 30 min at 37°C. To avoid degradation during electrophoresis, the sample was treated with $NaBH_4$ to reduce the AP sites. An aliquot (13 µl) was then mixed with 17 µl stop solution, and 15 µl were loaded into the 6 x 1.5 mm wells of a urea-containing 20 % polyacrylamide gel. The electrophoresis was run at 600 volts for 3 h. The two radioactive regions, one with pdT$_8$d(-)dT$_7$, and the other with pdT$_8$ and shorter oligonucleotides, were cut out, put in a scintillation mixture and counted. No degradation of the substrate 3' end, that might have interfered with the measurement of the AP endonuclease activity, occurred even with the highest exonuclease III concentrations. From the graphic representation of the results presented in Table I, one concludes that 10,000 endonuclease IV arbitrary units have the same AP endonuclease activity as 43 exonuclease III arbitrary units. It must be underscored that, in these experiments carried out in the presence of Mg^{++}, no exonucleolytic degradation of pdT$_8$ was observed when endonuclease IV was used even at the highest concentrations; it was, of course, different with exonuclease III.

The 3'-phosphoglycolatase activity was measured in the following way. A mixture of 5 µl pdT$_{20}$-[^{32}P]phosphoglycolate (11 pmol nucleotide) solution, 1 µl pdA (290 pmol nucleotide) solution, 25 µl Hepes buffer, 10 µl of diluted enzyme solution and water to make 50 µl, was incubated 30 min at 37°C. Were then added 50 µl 1 % phosphoglycolic acid aqueous solution, 400 µl 250 mM Tris.HCl, 25 mM

Table I : AP endonuclease and 3'-phosphatase activities of exonuclease III and endonuclease IV.

AP endonuclease	exo III	units		3	16	80	400	2000
		%		1.2	9	48	69	71
	endo IV	units		1250	2500	5000	10000	20000
		%		2.4	7	15	25	47
3'-phospho-glycolatase	exo III	units		1	2	4	10	20
		%	Mg	19	35	51	65	69
			EDTA					0
	endo IV	units		313	625	1250	2500	5000
		%	Mg	9	13	35	44	53
			EDTA			10	18	24

The enzyme units are arbitrary : 10 µl of the stock solution of either enzyme was said to contain 100,000 units; the values in the Table take account of the dilution factors. To measure the AP endonuclease activity, [5'-^{32}P]pdT$_8$d(-)dT$_7$, hybridized to polydA, was incubated in Hepes buffer containing Mg^{++} with exonuclease III or endonuclease IV; the [5'-^{32}P]pdT$_8$ released is given in percents of the total radioactivity. To measure the 3'-phosphoglycolatase activity, pdT$_{20}$-[^{32}P]phosphoglycolate, hybridized to polydA, was incubated in Hepes buffer containing Mg^{++} or EDTA with exonuclease III or endonuclease IV; the released [^{32}P]phosphoglycolate is given in percents of the total radioactivity.

KH_2PO_4, 20 mM $Na_4P_2O_7$, pH 8.5, and 500 µl of a 1:1 suspension of activated charcoal in water. After shaking, the suspension was left on ice for 5 min, centrifuged and the radioactivity of the supernatant measured. Checks using HPLC were made to show that the Norit-unadsorbable radioactivity was in phosphoglycolate. From a graphic representation of the results presented in Table I, one concludes that, when compared with exonuclease III, a 1,000-times more arbitrary units of endonuclease IV are needed to have the same 3'-phosphoglycolatase activity.

Comparison of the two sets of results indicates that, for the same AP endonuclease activity, the 3'-phosphoglycolatase activity of exonuclease III is about four times higher than that of endonuclease IV.

Effect of Mg^{++} :

The experiments using pdT$_{20}$-[^{32}P]phosphoglycolate were carried out as above in Hepes buffer except that 10 mM $MgCl_2$ was replaced by 1 mM EDTA. The 3'-phosphoglycolatase activity of exonuclease III was completely suppressed, whereas that of endonuclease IV was decreased about four-fold (Table I). The enhancement, by Mg^{++}, of the 3'-phosphoglycolatase activity of endonuclease IV was not due to a contamination with exonuclease III; indeed, in one step of the endonuclease IV preparation, exonuclease III was destroyed by heating at 65°C [3],

and we have shown (see above) that, in the presence of Mg^{++}, the endonuclease IV solution had no exonucleolytic activity.

DISCUSSION

In DNA, 3'-phosphoglycolate ends are hydrolyzed by exonuclease III [7] and endonuclease IV [10]. We confirm both results in this work. Endonuclease IV can be induced in Escherichia coli by agents that give rise to 3'-phosphoglycolate ends [11]. Since the repair of strand gaps limited by 3'-phosphoglycolate must begin with the hydrolysis of 3'-phosphoglycolate into 3'-hydroxyl that can prime DNA synthesis, our objective was to decide the relative importance of exonuclease III and endonuclease IV for this first repair step in the induced bacterium.

When E.coli is maximally induced, the AP endonuclease activity of endonuclease IV is about equal to that of endonuclease VI (exonuclease III) [11]. In this work, we show that, in the presence of Mg^{++}, for the same AP endonuclease activity, the 3'-phosphoglycolatase of exonuclease III is about 4 times more active than that of endonuclease IV. It would thus seem that, even in the induced bacterium, endonuclease IV has only a small part (20 %) of the 3'-phosphoglycolatase activity. In the non-induced organism, this part might drop to around 2 %. Of course, the Hepes buffer is not the cellular sap and the possibility of the presence of factors enhancing the relative 3'-phosphoglycolatase activity of endonuclease IV cannot be excluded, so that the real biological situation might be different.

Ionizing radiations produce strand breaks limited by 3'-phosphoglycolate ends and xth^- mutants of E.coli show only a low increase of X-ray sensitivity [8]. This suggests that the endonuclease IV 3'-phosphoglycolatase activity is sufficient to protect the bacterium unless the mutated exonuclease III has kept enough 3'-phosphoglycolatase activity although it has lost its other functions, or unless there is, in E.coli, a third 3'-phosphoglycolatase yet to be discovered.

We have also investigated the importance of Mg^{++} for these 3'-phosphoglycolatase activities. The divalent cation is absolutely necessary for exonuclease III, but it also considerably increases the 3'-phosphoglycolatase activity of endonuclease IV.

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