Multiple DNA repair activities for 3'-deoxyribose fragments in *Escherichia coli*

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Abstract

Escherichia coli contains multiple enzymes that hydrolyze deoxyribose fragments (phosphoglycolaldehyde, PGA) from the 3' termini of a synthetic DNA substrate. The major such activities are the main bacterial apurinic endonucleases, exonuclease III and endonuclease IV. In a double mutant deficient in both of these oxidation repair enzymes, Mg⁺⁺-dependent 3'-PGA diesterase was detected at 3% the level found in wild-type bacteria. Gel filtration fractionated this residual diesterase activity into two peaks of M_r 40,000-52,000 (Pool A) and M_r 22,000-30,000 (Pool B) with differing abilities to remove 3'-phosphates from DNA. These multiple repair activities were resolved in 3'-PGA diesterase activity gels. The exonuclease III and endonuclease IV bands were identified using the purified proteins and by their specific absence from strains defective for the respective structural genes. Gel filtration Pool B yielded two activity bands of apparent M_r 25,000 and 28,000, but Pool A did not form a new band in the activity gels. Incubation of activity gels in different transition metals or boiling of the samples before electrophoresis also served to distinguish the various activities. The possible identities of the novel *E. coli* 3'-PGA diesterases and the importance of multiple repair enzymes for 3' damages are discussed.

INTRODUCTION

Enzymes that remove 3'-deoxyribose fragments from DNA are important because such fragments block DNA repair synthesis and can be lethal if left in place (1). Deoxyribose is ruptured by a variety of chemical and physical agents that attack DNA. Strand breaks with 3' termini blocked by phosphoglycolate esters (3'-OPO₃-CH₂-COOH) have been identified in DNA exposed *in vitro* to the antibiotic bleomycin (2) or to ionizing radiation (3, 4). In the case of X- or γ-irradiation, this damage is mediated by the formation of partially reduced oxygen species in aqueous media (3). Such reactive forms of oxygen (superoxide radical: O₂^{-•}; hydrogen peroxide, H₂O₂; hydroxyl radical: •OH) are also formed during normal aerobic metabolism and by redox-cycling drugs such as paraquat (5, 6, 7). The ubiquity of the oxygen radicals that form blocked 3' termini in DNA demands the efficient removal of 3' fragments of nucleotides.

We have used a synthetic DNA substrate containing 3'-phosphoglycolaldehyde esters (3'-PGA) to detect and monitor the purification of enzymes from *Escherichia coli* (1, 8) and the yeast *Saccharomyces cerevisiae* (9) that remove such groups from DNA. This approach revealed that, under normal growth conditions, 95% of the cellular 3'-PGA

diesterase activity in *E. coli* is dependent on the *xth* gene, which encodes the enzyme exonuclease III (1). Exonuclease III is critical to the efficient recovery of *E. coli* from damage by H₂O₂ (1), probably because of the normally quantitative dominance of this enzyme as a 3' repair diesterase. Of the *xth*-independent enzyme activity, up to half is dependent on the *nfo* gene that encodes the enzyme endonuclease IV (8, 10). In addition, endonuclease IV is inducible up to 10-fold by agents that generate superoxide radical (11). The endonuclease IV 3'-PGA diesterase can be detected specifically in extracts of wild-type cells because of the enzyme's unique resistance to metal-chelating agents in the assay buffer (8).

We present here an investigation into the nature of the *xth*-/*nfo*-independent 3'-PGA diesterase activities of *E. coli*. The diesterase activity found in *xth nfo* double mutants results from several enzyme species that can be distinguished by their behavior during chromatography, by the pattern of products they release from DNA 3' termini, and through the use of 3'-PGA diesterase activity gels under various conditions. The activity gels show exonuclease III, endonuclease IV and two new 3'-PGA diesterases as distinct bands. We present evidence that *E. coli* contains a total of five distinct enzymes that can excise 3'-blocking fragments of deoxyribose from DNA. The significance of this multiplicity of enzymes is discussed, along with the possible identities of the newly described 3'-PGA diesterases.

MATERIALS AND METHODS

Bacterial strains. Strain AB1157 (*xth⁺nfo⁺*), the parent of the other strains used here, was a stock of this laboratory (1). Strains BW9109 ($\Delta xth nfo^{+}$), BW527 (*xth⁺nfo::kan*), BW528 ($\Delta xth nfo::kan$) and BW554 (BW9109/p-*nfo*; this strain contains the *nfo* gene on a multicopy plasmid) are described in Cunningham *et al.* (10) and were kindly supplied by B. Weiss (Johns Hopkins University School of Medicine, Baltimore, MD).

Chemicals and enzymes. Specially pure SDS (sodium dodecyl sulfate; BDH catalogue number 44244) for activity gels (12) was purchased from Gallard Schlessinger Chemical Mfg. Co. (Carle Place, New York). Ultrogel AcA54 was purchased from Pharmacia-LKB Biotechnology, Inc. [α -³²P]dCTP and [5-³H]dUTP were supplied by Amersham International. All other reagents were of the highest quality commercially available. Exonuclease III was from New England Biolabs, and the large (Klenow) fragment of DNA polymerase I from Bethesda Research Laboratories or Pharmacia-LKB. Uracil-DNA glycosylase from *E. coli* (1), phage T4 endonuclease V (1, 9) and *E. coli* endonuclease IV (8) were purified in this laboratory as described previously.

DNA substrates. The synthesis of DNA substrates containing 3'-PGA or 3'-phosphates (3'-P) has been described in detail (8, 9). The weight-average length of the typical substrate polymer used in these studies was ~1 kb; shorter polymers migrated significantly through the activity gels during electrophoresis. Longer polymers could be

made by decreasing the amount of [5-³H]dUTP present during DNA synthesis.

Enzyme assays. The 3'-PGA diesterase and 3'-phosphatase assays, and the paper chromatography of the reaction products were as described previously (8, 9). The assay mixtures contained the synthetic polymer with either 2 pmol 3'-PGA or 5 pmol 3'-P termini in 100 μ l 50 mM Hepes-KOH ("Hepes" is 4-(2-hydroxyl)-1-piperazineethanesulfonic acid), pH 7.6, 1 mM dithiothreitol, 50 μ g/ml BSA, and either 1 mM EDTA or 10 mM MgCl₂, as indicated. Crude bacterial extracts or purified enzymes were added as indicated to these mixtures and incubated at 37°C for the indicated times, followed by chilling on ice and adsorption with activated charcoal (Norit) in 0.4 M trichloroacetic acid (8, 9). Aliquots of the Norit-nonadsorbed material were analyzed by scintillation counting to determine the amount of released material. One unit of diesterase or monoesterase releases one pmol of product per minute under these conditions. For chromatography, the Norit-nonadsorbed fractions were extracted with ether to remove the tri-chloroacetic acid, applied to Whatman 3MM paper, chromatographed and the phosphate-containing compounds located as described (8, 9). The ³²P-containing compounds were quantitated by scintillation counting of 1-cm paper slices in 4 ml Scint-A fluor (Beckman Instruments, Inc.).

Preparation of cell-free extracts. Bacteria were grown to late log phase (OD₆₀₀=1) at 37°C in 1 l of LB broth (10 g/l Bacto-tryptone, 5 g/l Bacto yeast extract, 10 g/l NaCl) in the presence of the appropriate antibiotics, chilled and harvested at 10,000xg for 10 min at 4°C. The cell pellet was resuspended with 2 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM DTT, 0.3 mM NaCl) and lysed in a French pressure cell (two passes at 10,000 psi). The lysates were clarified by centrifugation at 20,000xg for 20 min, and the supernatants recovered and stored at -80°C. Protein concentrations in the extracts were determined by the method of Bradford (13) using BSA as the standard and ranged from 14 mg/ml to 24 mg/ml.

Gel filtration chromatography. A larger-scale extract of BW528 was prepared as described above, except that the cell pellet from two liters of culture (7.9 g) was resuspended in a larger volume (7.5 ml) of a modified buffer (lysis buffer as above also containing 1 mM EDTA and 10% sucrose). Eight ml of this extract was applied to a precalibrated column of Ultrogel AcA54 (2.5 cm x 200 cm) equilibrated with AcA54 buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM dithiothreitol, 0.5 M NaCl, 5% glycerol). The column was developed with this same buffer at 30 ml/h, and 5-ml fractions collected and assayed for 3'-PGA diesterase in the presence of 10 mM MgCl₂. Pools A and B eluted at 1.3-1.5 x V₀ and 1.7-1.9 x V₀, respectively. The two pools contained approximately equal amounts of diesterase activity, accounting for 30% of the total 3'-PGA diesterase applied to the column. After storage at -80°C, one-half of each pool was precipitated with (NH₄)₂SO₄ (4.5 g/ml) and redissolved in a minimal amount of AcA54

buffer diluted 1:1 with H₂O (Pool A, 2 ml; Pool B, 1 ml).

Samples of the two concentrated pools were rechromatographed on a smaller AcA54 column (1 cm x 26 cm) equilibrated with AcA54 buffer. Pool A: 0.30 ml applied to the column; Pool B: 0.33 ml applied to the column. The column was developed at 7 ml/h, and 0.35 ml fractions were collected and assayed for both runs. The rechromatographed Pool A yielded a single symmetric peak of activity centered on $1.4 \times V_0$, while Pool B yielded a single peak at $1.9\text{--}2.0 \times V_0$ (Fig. 1). The active peaks were pooled and stored at -80°C .

Activity gel analysis. The method described here is designed to detect renatured, active enzymes within a gel matrix after SDS-polyacrylamide gel electrophoresis and is based on procedures described by Spanos and Hübscher (12). Specially pure SDS was used for the gel and sample buffers. Samples for electrophoresis were mixed with one volume of twice-concentrated sample buffer [130 mM Tris-HCl, pH 6.8, 0.24 M β -mercaptoethanol, 2% SDS (freshly dissolved), 20% glycerol], heated at 37°C for three minutes unless specified otherwise, and loaded immediately on the gels. The resolving and stacking gels (15% acrylamide — 0.4% bis-acrylamide, and 5% acrylamide — 0.13% bis-acrylamide, respectively) were prepared as described by Laemmli (14), except that 5×10^5 cpm of the $3'\text{--}[^{32}\text{P}]\text{PGA}$ substrate was added per 10 ml of resolving gel mixture just before addition of the polymerization catalysts. The dimensions of the resolving gels were 15 cm x 8 cm x 0.08 cm. Electrophoresis was at 150 V for 4 to 5 h. Some polymer accumulates in the anode buffer chamber during electrophoresis, so that care should be taken in removing that buffer from the gel stand. Following electrophoresis, the SDS was removed by soaking the gels in three 30-min changes of 500 ml renaturation buffer (50 mM Tris-HCl, pH 7.5, 1 mM β -mercaptoethanol). Where indicated, 1 mM ZnCl₂ or 1 mM CoCl₂ was added to this buffer; when cobalt was present, the reducing agent was omitted from the buffer in order to avoid formation of a precipitate. The gels were then incubated 18 h at 4°C in 500 ml fresh buffer, followed by a further 24 h in fresh buffer at 37°C . A portion of the polymer may be lost from the gel after the 37°C incubation, probably by diffusion of the shorter species. The gels were then fixed by three 30-min washes, each time with 250 ml 5% trichloroacetic acid, 1% sodium pyrophosphate. Finally, the gels were stained with Coomassie blue R-250, destained and dried onto Whatman 3MM paper for autoradiography for 16 h at -80°C . The marker proteins visualized as stained bands were: phosphorylase B (92,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (22,000), and lysozyme (14,000).

RESULTS

Exonuclease III of *Escherichia coli* is the major cellular enzyme for the removal of 3'-blocking groups formed by H₂O₂ damage of DNA *in vivo* (1). *In vitro*, exonuclease III and endonuclease IV account for ~95% and $\leq 3\%$, respectively, of the *E. coli* cellular activity

Table 1. *E. coli* 3'-PGA diesterase and 3'-phosphatase activities independent of the *xth* and *nfo* genes.

Strain	Reaction condition	Activity (units/mg protein)	
		3'-PGA diesterase	3'-phosphatase
BW9109 (Δxth <i>nfo</i> ⁺)	EDTA	0.39	12.7
	MgCl ₂	2.6	2.4
BW528 (Δxth <i>nfo::kan</i>)	EDTA	<0.002	1.1
	MgCl ₂	2.2	17.7

Extracts were assayed using the synthetic 3'-PGA or 3'-phosphate substrates as described in the text. Some of the free phosphate released in the 3'-phosphatase assays may have been liberated by the sequential action of diesterases to release residual 3'-(2,3-didehydro-2,3-dideoxyribose-5-phosphate (present at about 35% of the 3'-termini in the polymer used in these experiments) and phosphatases acting on the released product (see text).

that hydrolyzes 3'-PGA esters from an artificial substrate (1, 8). Crude extracts of BW528, which is deficient in both exonuclease III (Δxth) and endonuclease IV (*nfo::kan*), contained most (85%) of the 3'-PGA diesterase detectable in the Δxth *nfo*⁺ strain BW9109 (Table 1). The total diesterase activity in BW528 was not changed by treating the cells with various levels of H₂O₂, or with different amounts of the superoxide generator paraquat, which induces endonuclease IV (14). Also unlike endonuclease IV, this residual 3'-PGA diesterase activity was undetectable when the enzyme assay buffer contained EDTA instead of MgCl₂ (Table 1). Substantial 3'-phosphomonoesterase activity is a feature of the 3'-PGA diesterases from *E. coli* (8,15) and yeast (16) thus far described in purified form. Strain BW528 also contained such DNA 3'-phosphatase that was largely dependent on Mg⁺⁺ (Table 1). The small amount of EDTA-resistant phosphatase that was detected in BW528 in these experiments could be due to other enzymes such as alkaline phosphatase. Paper chromatography revealed that the *xth*-/*nfo*-independent activity released both authentic PGA and authentic phosphate from DNA 3' ends (data not shown).

Two size classes of *xth*- and *nfo*-independent 3'-PGA diesterases. Gel filtration chromatography of an extract from the Δxth *nfo::kan* mutant BW528 yielded two broad activity peaks containing about equal amounts of 3'-PGA diesterase (data not shown; see below) that would correspond to globular species with M_r 40,000-52,000 (Pool A) and

Rechromatography of AcA54 Pools

Figure 1. Rechromatography of gel filtration Pools A and B. Samples were prepared and chromatographed on Ultrogel AcA54 as described in the text. The gel filtration fractions were assayed immediately for 3'-PGA diesterase in the presence of $MgCl_2$. Symbols: ●, rechromatographed Pool A; ○, rechromatographed Pool B.

22,000-30,000 (Pool B), respectively. The 3'-PGA diesterase recovered in these two pools together accounted for about 30% of the total activity applied to the columns. It seemed possible that the 3'-PGA diesterase activity of Pool A might be due to a dimeric form of the diesterase enzyme present in Pool B. In order to test this hypothesis, the gel filtration pools were concentrated by precipitation with ammonium sulfate, redissolved, and a portion of each was independently chromatographed on a smaller gel filtration column in the presence of 0.5 M NaCl. Each pool ran true, with the concentrated Pool A giving rise to a single peak of diesterase at $1.4 \times V_o$, while Pool B yielded a single broader activity peak at 1.9 - $2.0 \times V_o$ (Fig. 1). There was thus no indication of any significant interconversion of the Pool A and B activities (e.g., by dimerization), although this experiment does not rule out the possibility that the Pool B activity is a stable proteolytic product of the enzyme in Pool A.

The products released from different substrates by the two gel filtration pools were analyzed by paper chromatography. For both size classes of activity, authentic PGA was released from the 3'-PGA substrate, but Pool B contained relatively low amounts of 3'-phosphomonoesterase compared to the activity in Pool A (Table 2). The release of free phosphate from the synthetic polymer both by Pool A and by Pool B was accompanied by release of some of the residual 3'-ddR5P [3'-(2,3-didehydro-2,3-dideoxyribose-5-

Table 2. 3'-PGA diesterase, 3'-phosphatase and 3'-ddR5P diesterase activities of the *xth-/info*-independent enzymes of *E. coli*

<u>Enzyme fraction</u>	Activity (units/ml)	
	<u>3'-PGA diesterase</u>	<u>3'-phosphatase</u>
AcA54 Pool A	0.64	2.3
AcA54 Pool B	0.73	0.51
Endonuclease IV	158	545

The fractions were assayed and portions of the Norit-nonadsorbed material analyzed by paper chromatography as described previously (8). The 3'-phosphate substrate was only partially hydrolyzed by treatment with alkali in this experiment, and so contained about 25% 3'-ddR5P [3'-(2,3-didehydro-2,3-dideoxyribose-5-phosphate)]. Endonuclease IV was the purified protein (8) used as a dilute solution

phosphate)] present in this substrate (data not shown). These 3'-PGA diesterase fractions thus also contain significant amounts of 3'-diesterase for ddR5P. It is therefore possible that at least some of the free phosphate formed in the reactions with either Pool A or B was actually generated in two steps, with released ddR5P subsequently acted upon by a separate (non-specific) phospho-monoesterase. This question will have to be resolved by further enzyme purification. It is clear in any case that the relatively low phosphatase activity of Pool B contrasts with the 3'-phosphatase demonstrated in the well-characterized 3'-PGA diesterases exonuclease III (15), endonuclease IV (ref. 8; Table 2) and the yeast enzyme (16).

Activity gel analysis of *E. coli* 3'-PGA diesterases. In order to resolve the various *E. coli* 3'-PGA diesterase activities that were detected above, an activity gel method was developed. Samples of crude extracts, of the concentrated gel filtration samples, and of purified bacterial enzymes were separated by electrophoresis in SDS-polyacrylamide gels containing the 3'-PGA substrate, followed by treatments to allow renaturation and activity of the proteins (see **Materials and Methods**). In this system, polypeptides with 3'-PGA diesterase activity resulted in the release of [³²P]PGA from the substrate, revealed in autoradiographs as clear bands against a dark background due to the undigested substrate. This method consumes no more of the substrate polymer than a typical set of standard assays.

Figure 2. Activity gel analysis of 3'-PGA diesterases in *E. coli* mutants. Activity gel analysis was carried out as described in the text, with the samples incubated at 37°C prior to electrophoresis. The renaturation buffer did not contain added metals. The amounts of extract proteins loaded were: Lane 1, AB1157 (*xth*⁺ *nfo*⁺), 50 µg; lane 2, BW9109 (*Δxth* *nfo*⁺), 40 µg; lane 3, BW527 (*xth*⁺ *nfo::kan*), 30 µg; lane 4, BW528 (*Δxth* *nfo::kan*), 34 µg; lane 5, AcA54 Pool A, 0.02 units of diesterase; lane 6, AcA54 Pool B, 0.013 units of diesterase. Darker bands can be seen in the gel, which correspond to the positions of abundant proteins, including marker proteins with no demonstrated activity on DNA. This artifact may represent the accumulation of the substrate polymer at or behind protein bands during electrophoresis, or reduced loss of the substrate during the renaturation and activity incubations.

3'-PGA diesterase activity gels of crude extracts of various *E. coli* mutants revealed four distinct diesterase species. These included, in samples from wild-type cells, bands at apparent *M_r* 33,000, 28,000, and an unequal doublet at 25,000 (Fig. 2; positions calculated relative to standard marker proteins identified by staining with Coomassie blue). It should be noted that these numbers are not accurate measures of the polypeptide molecular masses, since some of the proteins appeared to remain partly folded during electrophoresis (see below). The 33-kDa band was due to exonuclease III, since this band was absent only from strains deleted for the *xth* gene (strains BW9109 and BW528: compare lanes 2 and 4 vs. 1 and 3 in Fig. 2). Furthermore, purified exonuclease III generated a single activity band at the same position (data not shown).

Analogous results showed that endonuclease IV accounts for the major 25-kDa species. That band was not observed in samples from the *nfo::kan* mutants BW527 and BW528 (compare lanes 3 and 4 to 1 and 2 in Fig. 2). Purified endonuclease IV formed a single band at this same position in activity gels (see below). Finally, samples from strain BW554 (which overproduces endonuclease IV from a multicopy plasmid) gave rise to a much stronger 25-kDa band than did a comparable amount of protein from a wild-type strain (see below).

Figure 3. Effect of transition metals on *E. coli* 3'-PGA diesterases in activity gels. The gels were run as described in the text, with the samples incubated at 37°C prior to electrophoresis. For each gel, the amounts of the samples loaded on the gel were as described in the Fig. 2 Legend, except where indicated. **A:** Renaturation buffer containing 1 mM CoCl₂. Lane 1, purified endonuclease IV (1 unit); lane 2, AcA54 Pool B; lane 3, AcA54 Pool A; lane 4, BW528 (Δxth *nfo::kan*); lane 5, BW527 (*xth⁺* *nfo::kan*); lane 6, BW9109 (Δxth *nfo⁺*). **B:** Renaturation buffer containing ZnCl₂. Lane 1, BW527; lane 2, AcA54 Pool B; lane 3, AcA54 Pool A; lane 4, BW554 (endonuclease IV overproducer), 8 μ g; lane 5, BW528; lane 6, BW9109; lane 7, AB1157 (*xth⁺* *nfo⁺*).

In all of the strains examined there remained two 3'-PGA diesterase activity bands, at M_r 28,000 and 25,000 (Fig. 2). Both of these bands were considerably fainter than those formed by either exonuclease III or endonuclease IV in wild-type cells. These new activities were both present in gel filtration Pool B, with only a small amount of the 28-kDa activity included in Pool A (Figs. 1 and 2). No band in the region corresponding to M_r 40-52 kDa was observed in any of the samples, even after longer autoradiography exposures to darken the background above M_r 40,000. Evidently, the Pool A diesterase does not appear in these activity gels.

Heat- or metal-sensitivity of diesterases in activity gels. The appearance of the individual 3'-PGA diesterases as bands in the activity gels could be modulated by altering either the treatment of the samples before electrophoresis or by modifying the renaturation buffer. When the renaturation buffer contained CoCl₂ (Fig. 3A) or ZnCl₂ (Fig. 3B), the exonuclease III band was not observed in lanes containing samples of *xth⁺* strains. In contrast, endonuclease IV activity was not inhibited by ZnCl₂, producing a strong 25-kDa band that was dependent on the *nfo⁺* gene (Fig. 3B). This same band intensity was obtained using only 8 μ g of extract protein from strain BW554, an overproducer of endonuclease IV, in contrast to the 50 μ g from wild-type cells needed to form a band of similar intensity (compare lanes 4 and 7 in Fig. 3B; each lane contains approximately 0.025 unit of endonuclease IV activity). This difference is consistent with the overproduction of

Figure 4. Effect of sample heating on *E. coli* 3'-PGA diesterases in activity gels. The amounts of the samples are as indicated in the Fig. 2 and 3 legends. All the samples were heated 5 min at 100°C prior to loading on the gel, with the exception indicated. Lane 1, purified endonuclease IV, 37°C incubation; lane 2, AB1157; lane 3, BW9109; lane 4, BW527; lane 5, Aca54 Pool A; lane 6, Aca54 Pool B.

endonuclease IV reported previously for strain BW554 (8, 11). One unit of purified endonuclease IV 3'-PGA diesterase activity generated only a faint band in gels incubated in CoCl_2 (Fig. 3A, lane 1), compared to the strong band seen for this amount of enzyme in renaturation buffer without added metals (Fig. 4, lane 1). When such large amounts of purified endonuclease IV were loaded on the activity gels, some band smearing (Fig. 4) and the occasional presence of fainter secondary bands at higher M_r (Fig. 3B and data not shown) could be seen. These species could result from either contaminants in the preparation or more unfolded forms of endonuclease IV itself. The intensity of the 25-kDa band generated by the *nfo*⁺ extract samples in the presence of cobalt (Fig. 3A) is probably due to both the *xth*-/*nfo*-independent 25-kDa diesterase, which does not appear to be inhibited by cobalt, and to the small amount of endonuclease IV activity observed in this buffer. The 28-kDa *xth*-/*nfo*-independent species was observed in the presence of CoCl_2 (Fig. 3A) but not ZnCl_2 (Fig. 3B). The 25-kDa activity persisted under both conditions.

The effect of heating the samples to 100°C prior to electrophoresis on the ability of the various diesterases to form bands in the activity gels provided a further means to distinguish between these activities (Fig. 4). The endonuclease IV band was largely lost upon the higher-temperature treatment. The relatively weak 25-kDa band present after 100°C treatment (Fig. 4, lanes 2-6) was likely due to the *xth*-/*nfo*-independent 25-kDa diesterase rather than some residual endonuclease IV activity, since this band was present in a sample from the *nfo::kan* mutant BW527. 100°C treatment of the samples prior to electrophoresis did not diminish the exonuclease III band. The 28-kDa *xth*-/*nfo*-

Table 3. Characteristics of 3'-PGA diesterases in *E. coli*.

Enzyme	Structural gene	P'ase/diesterase ratio	Present in activity gels after treatment with/at:			
			37°C	100°C	ZnCl ₂	CoCl ₂
Exonuclease III	<i>xth</i>	3.0	+	+	—	—
Endonuclease IV	<i>nfo</i>	3.4	+	—	+	+ / -
AcA54 Pool A	unknown	3.6	—	—	—	—
Pool B: 28-kDa	unknown	Pool B: 0.7	+	+	+	—
Pool B: 25-kDa	unknown		+	—	+	+

Abbreviation: P'ase is 3'-phosphodiesterase. The "P'ase/diesterase" ratios are the ratios of 3'-phosphatase to 3'-PGA diesterase activities for the various samples (see also Table 2). "Pool" indicates that this ratio is known only for the total activity in Pool B, but not for the individual enzymes. The phosphatase diesterase ratio for exonuclease III is an average number from a series of experiments over 3 years (A. Johnson and J. Levin, unpublished data).

independent diesterase was also unaffected by heating before electrophoresis, while the 25-kDa diesterase formed a weaker band following the high-temperature treatment. The characteristics that distinguish the various *E. coli* 3'-PGA diesterases are summarized in Table 3.

DISCUSSION

E. coli is equipped with a variety of enzymes that can remove *in vitro* 3'-deoxyribose fragments that block DNA 3'-termini. We have distinguished five such activities, based on genetic and biochemical analysis, as summarized in Table 3. The two known 3' repair enzymes are exonuclease III (encoded by the *xth* gene) and endonuclease IV (the *nfo* gene product). These enzymes have clear *in vivo* roles in repairing oxidative DNA damages (1,10). Endonuclease IV is also notable for its inducibility by agents that generate O₂^{•-} (11), and the enzyme thus seems to play a special role in repairing certain classes of oxidative DNA damage whose identities are not yet clear (10). No induction by oxidative mutagens has been detected for exonuclease III (17) or the novel 3'-PGA diesterases (this work). The several *xth*-/*nfo*-independent activities have been distinguished by their behavior during gel filtration chromatography and in 3'-PGA diesterase activity gels, and by their different 3'-PGA diesterase/3'-phosphatase ratios. Although the actual identities and substrate specificities of the new diesterases remain to be established, this multiplicity of activities could constitute an evolutionary response to both the intrinsic (7) and the

environmental production of oxygen radicals and the variety of damages they form in DNA.

The behavior of the various enzymes in 3'-PGA diesterase activity gels warrants comment. Exonuclease III and endonuclease IV both have polypeptide molecular weights near 30,000 (8, 15), yet only exonuclease III forms a band in activity gels near M_r 30,000, while the dominant endonuclease IV activity band is located at M_r 25,000. A likely explanation for this observation resides in the physical properties of endonuclease IV (8). The enzyme is very heat-stable in the absence of metal-chelating agents and probably retains a substantial amount of structure under mildly denaturing conditions, such as those used to treat the samples for activity gel analysis. A protein that retains some secondary and tertiary structure is expected to migrate faster during gel electrophoresis than is a completely denatured and therefore less compact polypeptide. Indeed, no activity band was formed when endonuclease IV was subjected to stronger denaturing conditions (heating in SDS at 100°C prior to electrophoresis; Fig. 4). Exonuclease III seems to be more reversibly denatured, since the intensity of the bands formed by that enzyme was not changed by the high-temperature treatment. It seems likely that exonuclease III does not renature completely, however, because the intensity of its activity band was always much less than that of endonuclease IV after 37°C treatment, even though the normal cellular concentrations of the two enzymes differ by about 20-fold (1, 8). It is also possible that some key cofactor for exonuclease III might have been omitted from the renaturation buffers, although the addition of 10 mM $MgCl_2$ did not enhance the exonuclease III band (data not shown). These considerations therefore preclude using activity gel measurements to assign either precise molecular weights or quantitative amounts of diesterase activities within a given extract. However, the relative amount of a given enzyme in different extracts can be assessed by the activity gel method.

The cofactor requirements of the various proteins are also not evident from the activity gels. Both exonuclease III and the *xth-/nfo*-independent activities require Mg^{2+} in solution (Table 1; ref. 8, 15) but not in the activity gels. As seen with exonuclease III, the addition of 10 mM $MgCl_2$ to the renaturation buffer did not intensify the 28-kDa or 25-kDa bands (data not shown). It may be that traces of Mg^{2+} or another metal are present in the gel or the renaturation buffer in a quantity sufficient to support the activities of exonuclease III and the *xth-/nfo*-independent enzymes. Alternatively, the magnesium requirement of these enzymes might be a non-mechanistic one (i.e., unrelated to the hydrolytic mechanisms of the enzymes) that is relieved in the gel matrix. The presence of transition metals during renaturation did have differential effects on the activity bands, with exonuclease III inhibited by both $ZnCl_2$ and $CoCl_2$, endonuclease IV largely inhibited by $CoCl_2$, and the 28-kDa activity inhibited by $ZnCl_2$ (Table 3). The inhibition by transition metals of the activities could reflect competition for the normal metal-binding sites on these proteins. Although native endonuclease IV does not exhibit a cofactor requirement, the

enzyme is inactivated by metal-chelating agents (8) and can be reactivated efficiently in solution by ZnCl_2 or certain other transition metals (J. Levin and B.D., unpublished data). Perhaps endonuclease IV retains its native metal cofactor under the activity gel conditions.

The 3'-PGA diesterases purified from yeast (9, 16) and partly purified from HeLa cells (unpublished data) did not form detectable bands in the activity gels described here. Although the yeast enzyme is strongly stimulated in solution by metals such as cobalt or zinc (9), the inclusion of either metal in the renaturation buffers did not reveal activity bands for either the purified or crude yeast 3'-PGA diesterase. Similarly, the inclusion of 10 mM MgCl_2 did not allow formation of an activity band by the magnesium-dependent HeLa 3'-PGA diesterase. The absence of activity bands corresponding to these eukaryotic nucleases was probably not due to the lighter background in the regions of the gels where they were expected to appear (M_r 40,500 (ref. 9) and ~50,000 (unpublished data), respectively, because longer autoradiography exposures failed to reveal such higher M_r bands.

The possible identities of the novel 3'-PGA diesterases are of interest. Neither the Pool A nor the Pool B activity seems to correspond to most known deoxyribonucleases of *E. coli* (18). Exonuclease III and endonuclease IV are ruled out on both genetic and biochemical grounds. The DNA polymerases and the exonucleases I, V (the RecBCD enzyme) and VIII are all substantially larger proteins than the Pool A or B activities. Exonuclease VII has a 54-kDa subunit, but that enzyme is resistant to EDTA and is specific for single-stranded DNA. Endonuclease I is a protein of only 12 kDa, while the apurinic endonucleases III and VII are both resistant to EDTA.

The Pool A activity, which does not appear as a distinct band on the activity gels, has some properties in common with the DNA deoxyribophosphodiesterase from *E. coli* recently described by Franklin and Lindahl (19). That enzyme has a native molecular weight of ~50,000, an absolute requirement for magnesium, and may remove 3'-terminal deoxyribose residues. Since this enzyme is not yet available in purified form, it is unclear whether the same deoxyribophosphodiesterase also hydrolyzes 3'-PGA.

The Pool B activities have some resemblance to *E. coli* DNA endonuclease V, which has a molecular weight near 20,000 (estimated by sedimentation) and a requirement for magnesium (20). Endonuclease V acts on a variety of small DNA damages, including apurinic and apyrimidinic sites (20), and seems to be capable of hydrolyzing a limited number of residues from nicks in duplex DNA (21). Thus, one of the Pool B enzymes could be identical to endonuclease V.

The 3'-PGA substrate has been useful for detecting and quantitating enzymes that remove sugar fragments that block DNA repair and replication after oxidative damage (1, 8, 9). The *E. coli* 3'-PGA diesterases exonuclease III and endonuclease IV have clear roles in repairing oxidative damages *in vivo* (1, 8, 10). The substrate specificity *in vitro* of the major 3'-PGA diesterase of *Saccharomyces cerevisiae* is similar to endonuclease IV of *E. coli*

(8, 17), indicating that the yeast and bacterial enzymes might have analogous intracellular functions. The question now arises of the true substrate specificity and cellular roles of the novel diesterases described here. The purification of these proteins and the isolation of enzyme-deficient mutants will be key steps to making these assignments. The availability of such a complex armory of enzymes to deal with oxidative damages probably reflects the variety of damages formed in DNA by free-radical attack and the need for their efficient repair.

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