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Relation between Escherichia coli endonucleases specific for apurinic sites in DNA and exonuclease III*

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ABSTRACT

Contradictory data have recently been published from two different laboratories on the presence vs absence of an intrinsic endonucleolytic activity of E. coli exonuclease III at apurinic sites in double-stranded DNA. It is shown here that an endonuclease activity of this specificity co-chromatographs exactly with exonuclease III on phosphocellulose and Sephadex G-75 columns, indicating that the endonuclease and exonuclease activities are due to the same enzyme. In addition, another E. coli endonuclease specific for apurinic sites exists, which can be separated from exonuclease III by the same chromatographic procedures.

INTRODUCTION

An endonuclease that specifically introduces single-strand breaks at apurinic sites in DNA has been purified from Escherichia coli cell extracts by Verly and co-workers[1,2]. They showed that the enzyme does not act on intact DNA or on alkylated DNA, but that it attacks at the apurinic sites in DNA which slowly appear as secondary lesions after alkylation. Goldthwait and co-workers[3,4] have also observed an E. coli endonuclease activity of this specificity, which was present in partly purified preparations of endonuclease II.

Several E. coli mutant strains, defective in endonuclease activity at apurinic sites in DNA, have recently been isolated[5-7]. These mutants, xthA⁻, have an increased sensitivity to the alkylating agent methyl methanesulfonate in comparison with wild type bacteria, indicating that the endonuclease is active in DNA repair in vivo. Yajko and Weiss[5] made the unexpected observation that such mutants were simultaneously deficient in DNA exonuclease III, an enzyme characterized over 10 years earlier[8] and since then widely used as a reagent enzyme. Moreover, E. coli mutants

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isolated as deficient in exonuclease III were simultaneously defective in endonuclease activity at DNA apurinic sites[5]. Exonuclease III attacks double-stranded DNA at the 3´ends and releases mononucleotides, and it has no endonucleolytic action on native DNA free from apurinic sites. Weiss[9] further showed that the exonuclease III function and an endonuclease activity for apurinic sites in DNA co-purified, and that a homogenous enzyme preparation contained both activities. In apparent contrast to this work, Goldthwait and co-workers[4] recently reported that E. coli endonuclease for apurinic sites and exonuclease III could be physically separated from each other, and that an E. coli mutant lacking exonuclease III but containing normal activity of endonuclease for apurinic sites had been found. Consequently, they proposed that these two activities are due to two different enzymes, and further hypothesized that the assay method employed by Weiss to measure the endonuclease activity might have been invalid because of false positive results due to exonuclease activity.

In the work described above, it has often been tacitly assumed that E. coli cells only contain a single endonuclease for DNA apurinic sites. This notion has turned out to be incorrect. E. coli xthA$^-$ mutant strains, defective in the major endonuclease activity for apurinic sites, still contain a remaining endonuclease activity of this specificity, which accounts for a minor part of the total endonuclease activity at DNA apurinic sites of wild type cell extracts and for most of the activity of xthA$^-$ mutant cell extracts[6]. Thus, E. coli cells contain at least two distinctly different endonucleases acting at apurinic sites in DNA. The new enzyme has been termed E. coli DNA endonuclease IV, and it has recently been extensively purified and shown to be absolutely specific for apurinic sites and apyrimidinic sites in DNA[10]. Endonuclease IV does not require divalent metal ions for activity and is fully active in the presence of 10^{-3} M EDTA. In this respect, it differs from the major endonuclease for apurinic sites, which is strongly inhibited by EDTA, and the enzymes can be differentiated in this fashion. In addition, Gates and Linn have recently characterized two E. coli DNA endonucleases that cleave DNA containing several different types of lesions, apparently including apurinic sites[11,12].

In the present work, we have purified E. coli exonuclease III by the method of Jovin et al. [13], which is a standard procedure for the preparation of this enzyme. In addition to assays for exonuclease III, we have also performed assays for endonuclease activity at apurinic sites in DNA, both in the absence and in the presence of EDTA. The method used to

evaluate endonuclease activity at apurinic sites was that devised by Verly et al.[1] and thus differs from the DNA gel assay employed by Weiss[9].

EXPERIMENTAL

E. coli exonuclease III was assayed by the method of Richardson et al.[8] and endonuclease for apurinic sites in DNA by the method of Verly et al.[1] with minor modifications[14]. The reaction mixtures for exonuclease III measurements (0.1 ml) contained 0.07 M Hepes·KOH, pH 8.0, 10^{-3} M $MgCl_2$, 0.01 M 2-mercaptoethanol, 10 µg bovine serum albumin, 2 µg native sonicated E. coli [^{32}P] DNA (40 000 cpm), and a limiting amount of enzyme, and they were incubated for 15 min at 37°. Reaction mixtures for endonuclease measurements (0.1 ml) contained 0.1 M NaCl. 0.05 M Hepes·KOH, pH 8.0, 2 x 10^{-3} M trisodium citrate, 10^{-4} M dithiothreitol, 50 µg bovine serum albumin, 2 µg yeast tRNA, 1 µg depurinated alkylated [^{32}P] DNA (20 000 cpm), and enzyme, and they were incubated at 37° for 30 min. In both kinds of assays, the reactions were stopped by chilling to 0° and addition of 0.1 ml cold 0.8 M perchloric acid. After centrifugation at 15 000 x g for 10 min, the radioactivity of the supernatants was determined. DNA 3'-phosphatase activity was measured by the method of Richardson and Kornberg[15], DNA polymerase I according to Jovin et al.[12], and carbonic anhydrase according to Henkens and Sturtevant[16].

E. coli exonuclease III is found both in the supernatant and the precipitate when crude cell extracts are treated with streptomycin sulfate[9], and better yields of the enzyme are obtained if a different method is used to remove nucleic acids from cell extracts. Here, we have followed the procedure of Sugden et al.[17], which involves removal of nucleic acids from extracts in an aqueous polyethylene glycol/dextran two-phase system, followed by step-wise hydroxyapatite chromatography of the protein fraction to remove the polymers. The enzyme was subsequently purified by ammonium sulfate fractionation, phosphocellulose chromatography, and gel chromatography according to Jovin et al.[12].

Crystalline human carbonic anhydrase was a generous gift from Dr. P. O. Nyman, Department of Biochemistry, Chalmers Institute of Technology, Gothenburg, Sweden.

RESULTS

An E. coli B cell extract was processed by two simple bulk fractionation steps and then chromatographed on phosphocellulose (Figure 1). Most

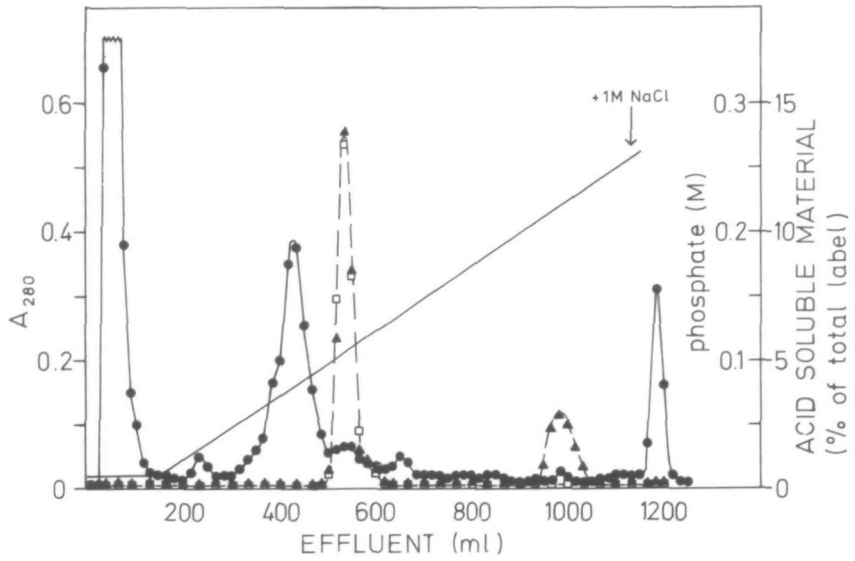

Figure 1. Phosphocellulose chromatography of a crude E. coli enzyme fraction[18]. Frozen E. coli B cells (200 g) were disintegrated in an X press at -25°, thawed, and extracted for 1 hr at 2° with 1000 ml of 5 M NaCl, 0.05 M Tris·HCl, pH 8.0, 10^{-4} M EDTA, and 10^{-4} M dithiothreitol. After clarification of the extract by centrifugation at 10 000 x g for 20 min, 200 ml 28% (w/v) Dextran T-500 (Pharmacia Ltd.) in 5 M NaCl and 200 ml 49% (w/v) polyethylene glycol 6000 in 2 M NaCl were added. After mixing, the two phases were separated by centrifugation, and the top (protein-containing) phase was washed once with fresh bottom phase. It was then dialyzed for 15 hr against 0.3 M NaCl, 0.02 M Tris·HCl, pH 7.5, 0.01 M 2-mercaptoethanol, 10^{-3} M K_2HPO_4, and applied to a column (5.3 x 18 cm) of hydroxyapatite (Hypatite C, Clarkson Chemical Co.) equilibrated with the same buffer. After washing of the column with the buffer, the adsorbed protein, which represented more than 90% of the applied protein, was eluted with 0.3 M potassium phosphate, pH 7.5, 0.01 M 2-mercaptoethanol. The eluate was supplemented with EDTA (10^{-4} M), and the protein fraction precipitating between 50% and 70% saturation with ammonium sulfate was recovered. The enzyme fraction was suspended in 25 ml of 0.02 M potassium, pH 6.6, containing 0.01 M 2-mercaptoethanol, and dialyzed against two changes of the same buffer. The protein (550 mg in 40 ml) was then applied to a 2x20 cm column of phosphocellulose (Whatman P-11) equilibrated with the same buffer, and after removal of non-adsorbed protein by washing, a linear gradient (2 x 500 ml, 0.02 M to 0.3 M phosphate, pH 6.6, containing 0.01 M mercaptoethanol) was employed to elute adsorbed proteins. Aliquots (1 µl) of indicated fractions were assayed for nuclease activities. ● ——— ● A_{280} measurements; □ ----- □ exonuclease III activity; ▲ – – – – ▲ endonuclease activity with partly depurinated DNA as substrate. Phosphate concentrations were conductimetrically determined.

of the protein did not adsorb to the column, but all detectable endonuclease activity for partly depurinated DNA as well as all exonuclease III activity was adsorbed. On elution with a linear salt gradient, a single peak of exo-

nuclease III activity but two different peaks of endonuclease activity for apurinic sites were observed. The first peak of endonuclease activity coincided exactly with the peak of exonuclease III activity, indicating that exonuclease III has an intrinsic endonucleolytic activity against partly depurinated DNA.

In the presence of citrate and simultaneous absence of added divalent metal ions, E. coli exonuclease III preparations do not exhibit exonuclease activity on DNA, but the associated endonuclease for apurinic sites remains active under the assay conditions employed here[14]. Thus, acid-soluble material is released from partly depurinated DNA in the form of large oligonucleotides, and a maximum of about 15% of the radioactivity of the standard DNA substrate employed can be released in acid-soluble form with an excess of enzyme. Equivalent results in this regard were obtained with the two different endonuclease activities eluted from the phosphocellulose column. The endonucleolytic activity observed in the exonuclease III-containing fractions consequently can not be ascribed to a false positive assay due to exonuclease activity, but reflects endonuclease action at apurinic sites in DNA. The present assay method was used in the initial characterization of an E. coli endonuclease for apurinic sites[1], and it has previously been shown to yield the same results as a more complicated but highly specific assay based on bacterial transformation with DNA containing apurinic sites in a direct comparison of the two methods[6].

The observed peak of exonuclease III activity co-chromatographed with a DNA 3'-phosphatase activity[15], as expected (data not shown). Further, DNA polymerase I was eluted at 0.12 M phosphate, immediately after the peak of exonuclease III activity, in agreement with observations by Jovin et al.[12]. The second peak of endonuclease activity for partly depurinated DNA, eluted at 0.22 M phosphate, did not contain detectable associated exonuclease activity, DNA phosphatase activity, or endonuclease activity for intact DNA. Further, the enzyme was unusually resistant to salt, with 65% of maximal activity remaining in the presence of 0.5 M NaCl, and it was more active at pH 8 than at pH 7. These properties serve to identify the enzyme as endonuclease IV[10]. Moreover, when the different column fractions were assayed for endonuclease activity on partly depurinated DNA in reaction mixtures supplemented with 10^{-3} M EDTA, the activity of the first peak was strongly inhibited (< 2% residual activity), while the second peak retained full activity. This is again consistent with the notion that the first peak represents the endonucleolytic function of exonuclease III, while the second peak represents endonuclease IV[10, 14]. In addition to the

two endonuclease activities observed here, E. coli cell extracts may contain other endonucleases active on partly depurinated DNA[11], which would not have been detected under the present experimental conditions.

In crude cell extracts, the endonucleolytic function of exonuclease III accounts for most of the total endonuclease activity on partly depurinated DNA, and endonuclease IV only for a minor proportion[6]. After purification by different types of chromatography, however, the relative proportion of the total endonuclease activity on partly depurinated DNA that is due to exonuclease III regularly decreases. This is because exonuclease III is a labile enzyme, so it is difficult to avoid considerable losses of enzyme activity during purification. In contrast, endonuclease IV is a stable enzyme that usually gives high yields in standard methods of chromatographic puri-

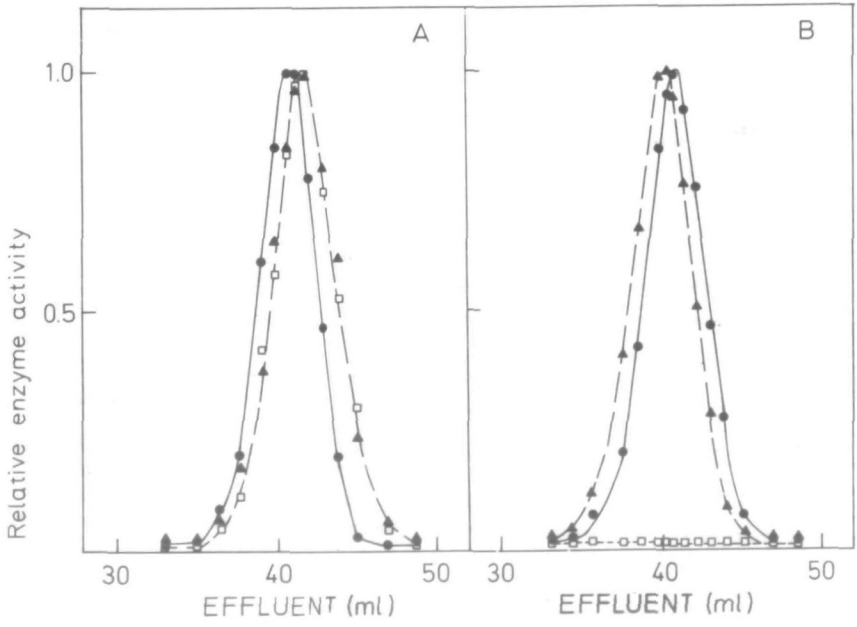

Figure 2. Gel chromatography of E. coli endonucleases acting at apurinic sites in DNA. The two peaks of endonuclease activity observed after phosphocellulose chromatography (Figure 1) were pooled separately and concentrated in an Amicon ultrafiltration cell equipped with a Diaflo PM10 membrane. Aliquots of the enzymes were mixed with a small amount of carbonic anhydrase, which served as an internal size marker, and chromatographed on a pre-calibrated Sephadex G-75 column (1.1 x 101 cm), equilibrated with 1 M NaCl, 0.05 M potassium phosphate, pH 7.4, 10^{-3} M dithiothreitol, 5% glycerol. The eluted fractions were assayed for endonuclease activity on partly depurinated DNA (▲ - - - - ▲), exonuclease III activity (□ - - - - □), and carbonic anhydrase activity (● ——— ●). A. Exonuclease III. B. Endonuclease IV.

fication. The relative proportions of the two activities in Figure 1 are consequently not representative of the relative amounts present in crude cell extracts.

The two different endonuclease activities observed here were further fractionated separately by gel chromatography on Sephadex G-75. Weiss[9] has observed that exonuclease III and the endonuclease activity associated with the enzyme co-chromatograph in such experiments. On the other hand, Goldthwait and co-workers[4] have reported that the endonuclease for apurinic sites and exonuclease III can be partly separated by gel chromatography, and that the endonuclease is eluted before the exonuclease. Here, we have used an internal reference in all experiments that does not interfere with the various nuclease assays, crystalline human carbonic anhydrase (M_r = 29 000), to provide increased accuracy over the volume measurements. The data are shown in Figure 2. Exonuclease III was eluted immediately after carbonic anhydrase in co-chromatography experiments with a mixture of the two enzymes. The endonuclease activity for apurinic sites in DNA of the enzyme preparation, as well as the DNA 3′-phosphatase activity, co-chromatographed exactly with the exonuclease activity (Figure 2A). In contrast, endonuclease IV was eluted slightly before carbonic anhydrase (Figure 2B). The Stokes radius of human carbonic anhydrase is r_s = 2.3 nm, and from the present experiments we estimate that exonuclease III with its associated endonucleolytic activity has r_s = 2.2 nm, while endonuclease IV has r_s = 2.4 nm. Since endonuclease IV also has a higher sedimentation coefficient than exonuclease III[9, 10], it seems clear that there is a small but distinct molecular weight difference between these two enzymes.

DISCUSSION

The results presented here are in agreement with the data of Weiss[9], showing that *E. coli* exonuclease III has an intrinsic endonucleolytic activity on partly depurinated DNA, while they do not confirm the report of Kirtikar *et al.*[4] that *E. coli* exonuclease III can be purified free from endonuclease activity at apurinic sites. Employing a different assay method for endonuclease activity at apurinic sites than that employed in these previous studies, we have now shown that an endonuclease activity co-chromatographs exactly with exonuclease III in phosphocellulose chromatography and gel chromatography experiments. Gossard and Verly[19] have briefly reported similar data, and S. Rogers and B. Weiss (pers. commun.) as well as S. Linn (pers. commun.) have recently shown that apparently

homogenous preparations of exonuclease III catalyze the formation of single-strand breaks in covalently closed circular DNA molecules containing apurinic sites. Further, extensive genetic studies have provided independent evidence for the notion that E. coli exonuclease III and the major endonuclease for apurinic sites in DNA are the same enzyme[5-7], and we consider this point settled. The present data may also provide a rationale for some of the observations of Goldthwait and co-workers[4], as E. coli cell extracts contain an endonuclease active on partly depurinated DNA that is separable from exonuclease III by ion exchange chromatography and gel chromatography.

It is finally noted that neither of the two enzymes described here has the ability to selectively degrade alkylated DNA[1,10], although they act at a common secondary lesion occurring after alkylation of DNA, i.e. apurinic sites. With regard to nomenclature, it would seem preferable that the major E. coli endonuclease activity for partly depurinated DNA is not given a separate name but that it is referred to as "the endonucleolytic function of exonuclease III", in analogy with the term "the 3'-phosphatase function of exonuclease III". E. coli endonuclease IV is clearly a separate enzyme and should retain its own name. The designation "endonuclease II" has most recently been used to describe an activity different from those discussed here[4]. Finally, terms such as "the apurinic endonuclease" or "endonuclease for apurinic sites" should not be used without further specification, as E. coli cells contain more than one enzyme of this type.

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