Identification of a new deoxyendonuclease from *Escherichia coli* that preferentially cleaves supercoiled plasmid DNA

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1. INTRODUCTION

Deoxynucleases of *Escherichia coli* have been thoroughly studied because of the current interest in genetic recombination where enzymatic cleavage of DNA strands is an essential step. Apart from site-specific restriction enzymes, a number of deoxyendonucleases of *E. coli* have been well-characterized which include endonuclease I [1], endonuclease III [2], endonucleases IV–VI [3–5] and *rec*BC endonuclease [6]. We wish to report here the isolation of a new endonuclease from *E. coli* which has been purified to apparent homogeneity. This enzyme readily makes a single double-strand cleavage in supercoiled plasmid DNA to generate a linear DNA-duplex but acts poorly on linear double-stranded DNA. Studies with this highly purified enzyme, which we tentatively name endonuclease X, show that its properties are distinct from those of the other well-characterized deoxyendonucleases of *E. coli* reported previously.

2. MATERIALS AND METHODS

2.1. *Bacterial strain*

The source of the enzyme is the strain *E. coli* U277, which is a laboratory isolate showing an ability to grow on salt–glucose synthetic medium and exhibiting drug-resistance against streptomycin and ampicillin. The strain restricts λ . K but not λ . B (the plating efficiency of λ . B and λ . K on the bacteria was 1 and 10^{-4}, respectively). It appears that the strain is a wild-type λ-sensitive derivative of *E. coli* B; the drug resistance character could be due to a plasmid, but this point has not been verified.

2.2. *Preparation of endonuclease X*

Endonuclease X was found in the periplasmic proteins of *E. coli* U277 which could be easily obtained by osmotic shock of the cells following the procedure in [7]. The shocked fluid from 2 l cells, grown to early stationary phase in Luria broth at 37°C, was subjected to DEAE-cellulose chromatography (20 ml column vol.) using a 100 ml linear gradient of 0.0–0.3 M NaCl in EXBT buffer (KPO_4, pH 7.0, 10 mM; EDTA, 1 mM; 2-mercaptoethanol, 7 mM and Triton X-100, 0.15%). Forty fractions were collected and alternate fractions were assayed for endonuclease X activity. DEAE-cellulose fractions showing peak activity (34–36) were further purified by (a) blue Sepharose [11] and (b) hydroxylapatite chromatography. In blue Sepharose chromatography, half the volume of pooled fractions was diluted 3-fold with buffer A [0.02 M Tris–HCl (pH 7.6)–0.1 M $MgCl_2$–0.01 M 2-mercaptoethanol] and loaded onto a small column (0.5 ml), and the activity

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eluted successively with 2 ml portions of 1 M NaCl in buffer A. After adding bovine serum albumin (100 μg/ml) to the pooled active fractions, it was dialyzed against EXBT containing 5% glycerol (BS-fraction). In hydroxylapatite chromatography, the other half of the pooled DEAE-cellulose fractions was diluted 3-fold with PME buffer [0.01 M KPO_4 (pH 7.0)–14 mM 2-mercaptoethanol–1 mM EDTA] and loaded onto a column (1 ml bed vol.) and the proteins were eluted batchwise with 1 ml portions of increasing concentrations of K-phosphate (pH 7.0) in PME. The maximum activity was obtained in the second fraction of 0.25 M K-phosphate eluate (HAP fraction, 40 μg protein/ml).

2.3. *Assay of endonuclease X*

A typical incubation mixture, in a total volume of 40 μl contained 0.5 μg pBR322 DNA, 25 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 5 mM Mg^{2+} and varying amounts of endonuclease X. The incubation was carried out at 37°C. Incubation mixtures, after termination of activity by adding 20 μl of a solution containing 50 mM EDTA, 30% (w/w) sucrose and 0.005% bromophenol blue, were directly loaded onto 0.7% agarose gel tubes and electrophoresed in Tris–borate buffer [8]. We used tube gels instead of slab gels for DNA electrophoresis since the former gave better pictures of DNA bands, after ethidium bromide staining, when they were illuminated by a hand-model UV-lamp from one side. Formation of linear (form III) and nicked circular DNA (form II) from supercoiled plasmid DNA (form I) was visually compared for endonuclease X activity.

2.4. *Plasmid and bacteriophage DNA purification*

pBR322 and ColE1 plasmid DNAs were prepared by the hydroxylapatite chromatographic method in [10]. λ DNA was prepared from *E. coli* K12 C600 ($\lambda CI_{857}S_7$) and purified by banding in a CsCl gradient.

3. RESULTS AND DISCUSSION

Fig.1a demonstrates the endonuclease X activity in the DEAE cellulose fractions (22–40) on pBR322 plasmid DNA which shows the production of a discrete linear DNA fragment. When λ DNA replaced pBR322 as substrate, none of the DEAE-cellulose fractions could generate discrete DNA fragments indicating the absence of restriction endonuclease-like activity in the preparation (not shown). The endonuclease activities of the highly purified fractions from blue Sepharose and hydroxylapatite chromatographies are shown in fig.1b,c. The hydroxylapatite chromatography with K-phosphate buffer apparently yielded a homogeneous protein as can be judged from the SDS–polyacrylamide slab gel electrophoresis which showed only a single band for the HAP fraction (fig.2). This band corresponded to M_r 49000. The active BS fraction, however, showed two protein bands, one of which corresponded to the single band of the HAP fraction. Therefore, we performed our subsequent experiments with the HAP fraction to study the properties of endonuclease X.

The endonuclease activity showed an absolute dependence on a divalent cation, such as Mg^{2+} or Ca^{2+}, and a broad pH optimum between 8 and 9. We therefore routinely used 5 mM Mg^{2+} in 0.025 M Tris–HCl buffer (pH 8.0) for the assay of the enzyme where the activity was optimum.

Kinetic experiments with the purified endonuclease showed, as in [12,13], that initially only the form II of pBR322 was produced (fig.3, lane 2) when the enzyme concentration was kept low, but at higher concentrations of the enzyme or when the incubation time was prolonged, the linear form of pBR322 could be seen as the major product (fig.3, lanes 5,7). This is further corroborated by the enzyme action on form I of pBR322-dimer which was also present as a minor component in our preparation. This was converted to form II and form III of the dimer as can be seen in fig.3.

In order to demonstrate that the product of endonuclease X activity on supercoiled DNA is a linear DNA-duplex of unit length, we digested ColE1 and pBR322 plasmid DNA with *Eco*RI and *Bam*HI, respectively (each having a single restriction site on the plasmid) yielding linear DNA-duplex (fig.4a, lane 5; fig.4b, lane 2) and compared their electrophoretic mobility in agarose gel with that of the products generated by endonuclease X from the same plasmids (fig.4a, lane 6; fig.4b, lane 4). As the linear products of the restriction enzymes and endonuclease X from the same plasmid migrated at the same rate, it can be concluded that the endonuclease also makes a

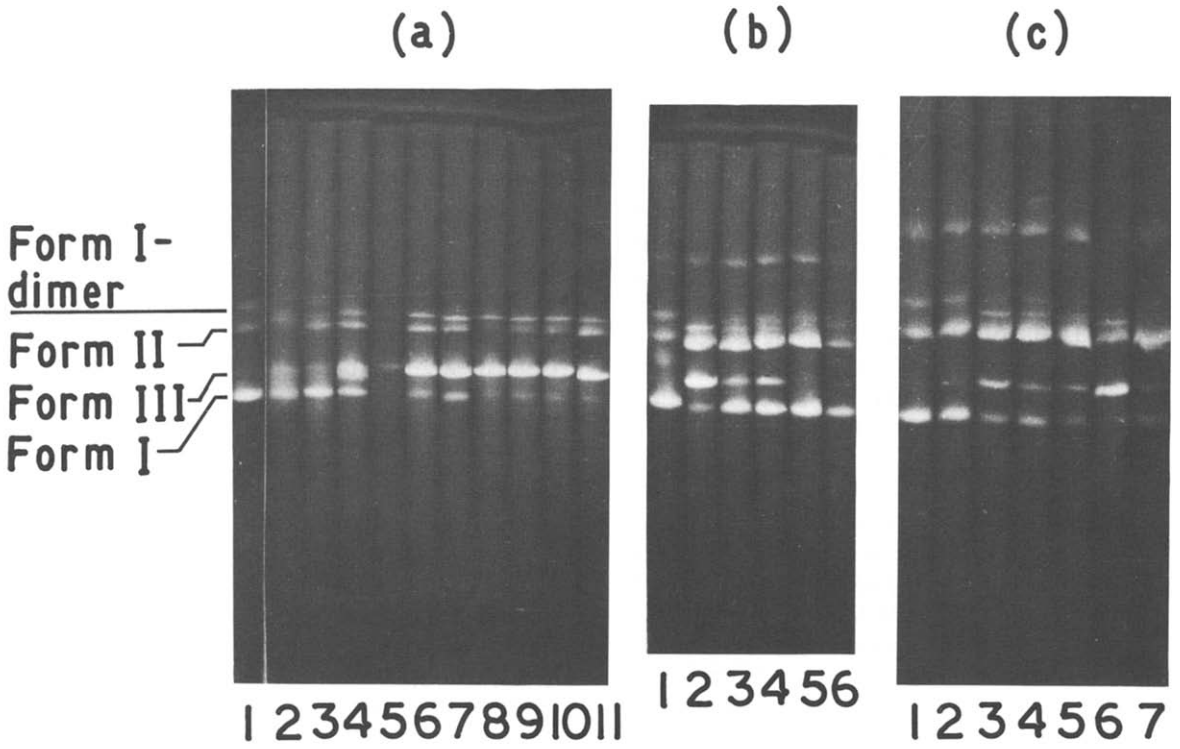

Fig.1. Assay of endonuclease X activity in various enzyme fractions. An aliquot from each fraction was added into the assay mixture containing pBR322 DNA and incubated for 1 h: (a) 5 μl aliquots of alternate DEAE cellulose fractions from 22–40 were assayed, lanes 2–11 – (1) control (i.e., without enzyme fraction), in addition to the major band (form I), small amounts of form II and form I-dimer of pBR322 plasmid were also present; (b) 20 μl aliquots of blue Sepharose fractions were assayed – (1) control; (2) load fraction; (3–6) four successive 1 M NaCl eluate fractions; (c) 5 μl aliquots of hydroxylapatite fractions were assayed – (1) control; (2–4) three successive 0.1 M KPO_4 (pH 7.0) eluate fractions; (5–7) three successive 0.25 M KPO_4 (pH 7.0) eluate fractions.

single double-strand cleavage in the plasmids, generating a unit length linear DNA-duplex.

That endonuclease X is not like a typical restriction enzyme was evident from two separate experimental results:

(i) It could not generate discrete linear fragments when acting on the bacteriophage λ DNA (fig.4a, lane 3);

(ii) It failed to show any double-strand cleavage activity on linearized pBR322 and ColE1 DNA produced by the restriction enzymes *Bam*HI and *Eco*RI respectively (fig.4b, lane 3; fig.4c, lane 3), where any cleavage site specific for endonuclease X should be present.

In addition, when linearized ColE1 (by *Eco*RI) or λ DNA was added along with supercoiled pBR322 to the incubation mixture, the linearization of pBR322 was not appreciably inhibited by either ColE1 or λ. The enzyme activity on λ DNA shows slight smearing on the gel which suggests that endonuclease X is associated with some nicking activity. This, however, is not unexpected because even very highly purified single-strand specific nucleases (e.g., S1) do show some non-specific nicking activity on double-stranded DNA [12].

Further, it was of interest to know whether the endonuclease has any preferential site of cleavage in pBR322. When the linear product from pBR322 formed by endonuclease X was subjected to further cleavage by *Bam*HI, we observed the production of at least three distinct bands shorter than the pBR322 linear product in agarose gel (fig.5). This result shows some site-specificity of the endonuclease in the double-strand cleavage reaction on supercoiled pBR322 DNA; had the enzyme

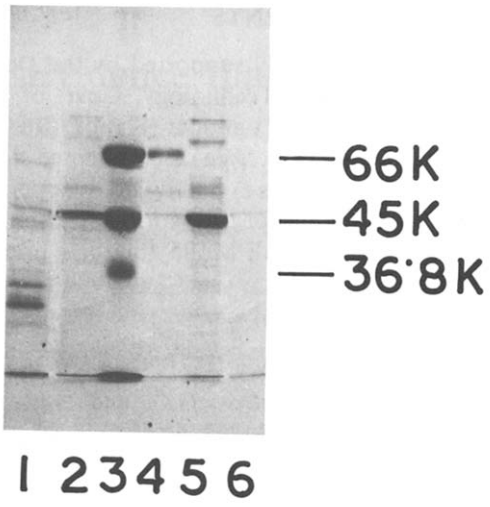

Fig.2. SDS–polyacrylamide gel electrophoresis of enzyme fractions at different stages of purification. Samples were prepared and electrophoresed in slab gels (10% polyacrylamide; 16 cm × 16 cm, 2 mm thick) according to the procedure in [9]: (1) osmotically shocked fluid (10 μl); (2) DEAE-cellulose fraction 36 (20 μl); (3) M_r markers; (4) BS fraction (20 μl, the topmost band is due to added bovine serum albumin in the BS fraction); (5) 0.1 M KPO_4 (pH 7.0) hydroxylapatite column eluate, second fraction (10 μl); (6) HAP fraction (20 μl).

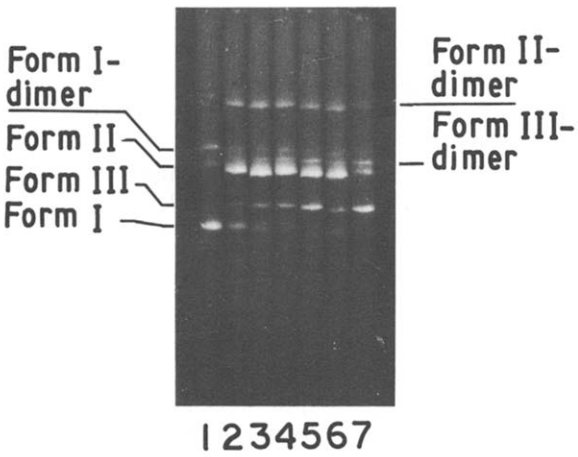

Fig.3. Demonstration of the formation of nicked circles (form II) as an intermediate during the enzymatic conversion of supercoiled pBR322 DNA (form I) to its linear form (form III): (1) control; (2,4,6) 20 min incubation with 1 μl, 2 μl and 3 μl of HAP fraction, respectively; (3,5,7) 40 min incubation with 1 μl, 2 μl and 3 μl of the HAP fraction, respectively.

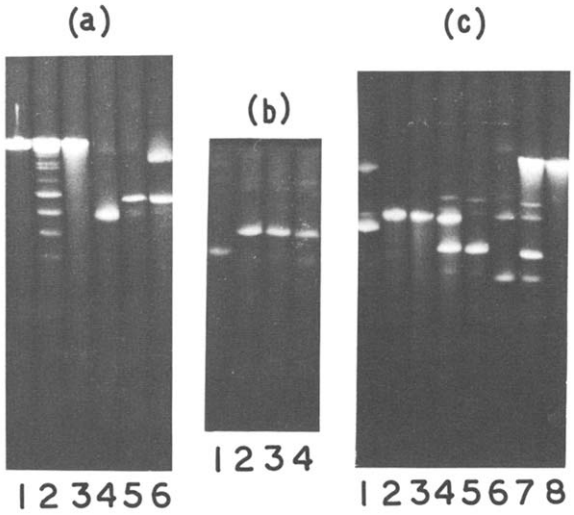

Fig.4. Evidence that endonuclease X makes a single double-strand cleavage in supercoiled plasmid DNA, and its lack of activity on linear DNA. All incubations were carried out for 1 h and 5 μl/assay tube of HAP fraction was aded, where used. Amounts of λ, pBR322 and ColE1 DNA used per assay tube were 0.8 μg, 0.4 μg and 0.5 μg, respectively. (a) Lanes 1 and 4, control tubes of λ and ColE1 DNA, respectively; lanes 2 and 5, λ and ColE1 respectively treated with *Eco*RI; lanes 3 and 6, λ and ColE1 respectively treated with HAP fraction. (b) Lane 1, pBR322 DNA (control); lane 2, pBR322 treated with *Bam*HI; lane 3, linearized pBR322 (by *Bam*HI) treated with HAP fraction; lane 4, pBR322 treated with HAP fraction. (c) Lanes 1 and 6, control tubes of ColE1 and pBR322 DNA, respectively; lane 2, ColE1 treated with *Eco*RI; lane 3, linearized ColE1 (by *Eco*RI) treated with HAP fraction; lane 5, pBR322 treated with HAP fraction; lane 4, a mixture of linearized ColE1 (by *Eco*RI) and pBR322, treated with HAP fraction; lane 8, λ treated with HAP fraction; lane 7, a mixture of λ and pBR322 treated with HAP fraction.

cleaved the supercoiled DNA randomly, the subsequent *Bam*HI treatment would show a smearing of the product in agarose gel. Though the enzyme appears to cleave at more than one site on supercoiled DNA, a double-strand break at one site seems to block further double-strand cleavage. Recently, certain endonucleases that preferentially cleave single-stranded DNA such as endonuclease SI, T7 gene 3 product [12] and micrococcal endonuclease [13] have been shown to generate a linear product by a single double-strand cleavage at certain preferred sites (single-stranded loops of potential

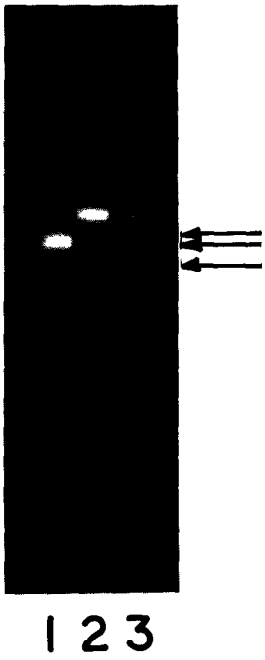

Fig.5. Presence of preferential sites in pBR322 DNA for cleavage by endonuclease X: (1) pBR322 DNA (1 μg), control; (2) pBR322 linearized by HAP fraction; (3) linearized pBR322 treated with *Bam* HI.

cruciform structures) in supercoiled pBR322 DNA. The similarity of activity of this endonuclease X from *E. coli* with that of the other deoxyendonucleases on supercoiled DNA suggests that endonuclease X might act preferentially on single-stranded rather than on double-stranded DNA. The possibility that U277 harbours a drug-resistant plasmid and it determines the endonuclease X activity cannot be excluded at this stage.

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