The Release of Oligonucleotides by the *Escherichia coli* B Restriction Endonuclease

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SUMMARY:
The *Escherichia coli* B *(Eco* B) restriction endonuclease releases approximately 75 nucleotides as acid-soluble oligonucleotides for each single-strand endonucleolytic scission that it catalyzes. This reaction, like the endonucleolytic cleavage, requires ATP, Mg^{++}, S-adenosylmethionine, and unmodified DNA containing appropriate specificity sites. Like the endonuclease reaction, the release of oligonucleotides terminates after roughly 5 minutes. The acid-soluble oligonucleotides have an average chain length of roughly 7, and an apparently random base composition.

INTRODUCTION:

The restriction endonuclease of *E. coli* B is a complex enzyme requiring ATP, S-adenosylmethionine, Mg^{++}, and DNA containing specific sequences which have not been modified by the complementary modification methylase (1-3). The enzyme appears to make only one endonucleolytic scission, then converts itself to a DNA-dependent ATPase which hydrolyzes ATP to ADP and inorganic phosphate for up to several hours (4,5). This enzyme is unlike most restriction nucleases in its specificity: It recognizes a unique sequence, but the sequence has no obvious symmetry (6) and it does not break the DNA at the specificity sequence, but more or less randomly (4,7,8). All that is known concerning the location of this cleavage is that the enzyme is reluctant to act upon, or to form duplex fragments smaller than roughly 1500 base pairs (4,9).

On studying the products of this enzyme we have observed another enzymic reaction--the formation of small, acid-soluble DNA fragments. This report presents evidence that these oligonucleotides are formed by the restriction enzyme, not by a contaminant. It also gives some characteristics of the fragments.

MATERIALS AND METHODS:

Restriction enzyme was the sucrose gradient fraction, unless otherwise indicated, and was prepared as described previously (1). One unit of enzyme causes 0.6 pmol of single-stranded DNA scissions (1). The preparations of ^{3}H- or ^{32}P-labelled DNA from *E. coli*, ^{3}H-labelled DNA from phage λ and [γ-^{32}P]ATP were described (1,10). Techniques used to characterize the oligonucleotides were as described by Goldmark and Linn (10).

Restriction reactions (70 µl) contained 70 mM Tris-HCl (pH 8.2), 7 mM $MgCl_2$, 0.5 mM DTT, 1.4 mM ATP ([γ-^{32}P]ATP was used for ATPase assays), 14 µM S-adenosylmethionine, 1.5 nmol DNA, and restriction enzyme. Incubations were for 15 min at 37°. ATPase and endonuclease assays were as described previously (1). DNA rendered acid-soluble was determined by adding 50 µl 5 mg/ml bovine

TABLE I

The release of acid-soluble DNA during the restriction reaction

Experiment	Assay conditions	pmol Acid-soluble nucleotide formed	Nucleotides released per DNA strand scission
I	complete	16.3	78
	- enzyme	<0.2	--
	- $MgCl_2$	<0.2	--
	- ATP	<0.2	--
	- S-adenosyl-methionine	<0.2	--
II	+ λ•O DNA	209	84
	+ λ•O DNA (denatured)	<25	--
	+ λ•B DNA	<25	--
III	+ 0.11 unit PA Frn. 4	4.9	75
	+ 0.14 unit PA Frn. 7	5.9	69
	+ 0.18 unit SG enzyme	8.6	78

^{3}H-labelled *E. coli* K DNA (10,600 cpm/nmol) and 0.35 unit of enzyme were used in Experiment I.

Reactions as in Materials and Methods, but scaled up to 700 µl, were used in Experiment II. Phage λ DNA grown on a non-modifying strain of *E. coli* (λ•O DNA) or grown on *E. coli* B (λ•B DNA) had 1360 cpm/nmol and 1210 cpm/nmol, respectively.

E. coli K DNA (10,200 cpm/nmol) and enzyme as shown were used in Experiment III. "PA" enzyme is that eluted from polyacrylamide gels. Fractions 4 and 7 were those containing the two separate peaks of enzyme activity separated by electrophoresis on these gels, and were obtained from the particular gel pictured and assayed in Figs. 1 and 2 of reference 1. "SG" enzyme is sucrose gradient enzyme.

serum albumin and 0.25 ml 7% trichloracetic acid to a chilled reaction, incubating for at least 10 min at 0°; then centrifuging for 5 min at 25,000 x g and counting an aliquot of supernatant (10).

RESULTS AND DISCUSSION:

Characteristics of the reaction: When DNA is exposed to the *E. coli* B (*Eco* B) restriction endonuclease, roughly 75 nucleotides are released as acid-soluble material per single strand scission. This reaction has the same cofactor requirements as the endonuclease: Mg^{++}, ATP, and S-adenosyomethionine (Table I). In addition, the acid-soluble material is observed only with DNA not containing the B modification; it is not formed from DNA which has received the appropriate modifying methylation by growth in an *E. coli* B host (Table I). In addition, the reaction is not observed with heat-denatured DNA (Table I and Fig. 2 below) upon which the restriction endonuclease does not act (2). Finally the release of acid-soluble material is essentially complete after 5-10 min (Fig. 1). This is the time course of endonucleolytic scissions noted for the restriction reaction (1). In this way the release of acid-soluble material has the same reaction characteristics as the endonuclease.

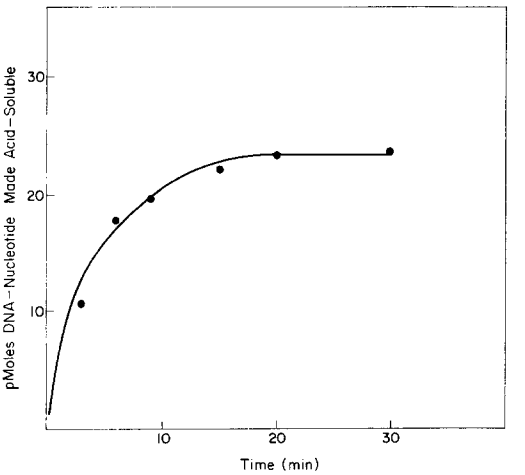

Figure 1. Time course for release of acid-soluble nucleotide. The reaction was as described in Material and Methods, but scaled up to 402 µl. ^{3}H-labelled *E. coli* K DNA (10,600 cpm/nmol) and 2.8 unit of enzyme were used. Fifty µl aliquots were removed at times shown and acid-soluble radioactivity was determined.

Association of the activity with the Eco B *restriction enzyme:* The final step of purification of the restriction enzyme is sedimentation through a sucrose gradient, which yields a preparation that is about 75% pure (1). All totally independent preparations of such material that were assayed (six) showed the same level of release of nucleotides relative to endonuclease activity. In addition, during the sedimentation, this activity co-migrated with the restriction enzyme as measured by its DNA-dependent ATPase (Fig. 2). (The activity releasing acid-soluble material from duplex DNA that trailed behind the ATPase is associated with a separate, contaminating activity which is removed in this step of the purification, that also acts on denatured DNA.)

When the *Eco* B restriction enzyme is subjected to electrophoresis upon polyacrylamide gels, two peaks of activity can be detected and eluted from the gel as homogeneous enzyme (Fig. 2 of reference 1). Each of these peaks of activity was assayed for the release of acid-soluble nucleotide, and

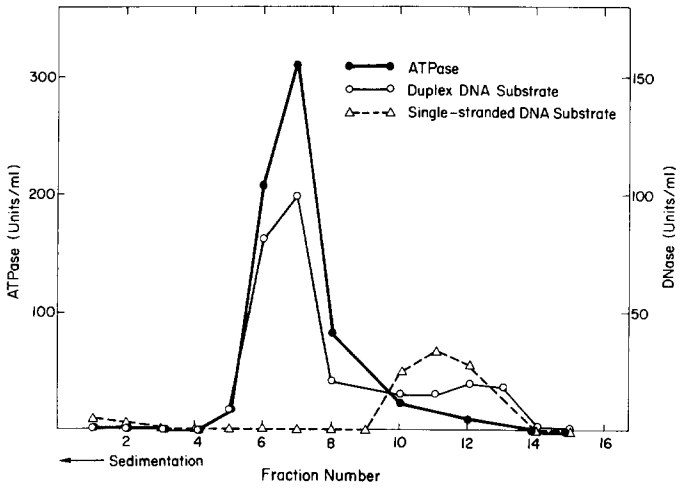

Figure 2. Preparative sucrose gradient sedimentation of restriction enzyme. DEAE-cellulose enzyme fraction was further purified by preparative scale sedimentation through a sucrose density gradient as described by Eskin and Linn (1). Gradient fractions were assayed as described in Materials and Methods with *E. coli* K DNA (^{3}H-labelled, 10,600 cpm/nmol for DNase assays), or, for ATPase assays, [γ-^{32}P]ATP (108 cpm/nmol). One unit of ATPase hydrolyzes 100 nmoles of ATP in 15 min (1), whereas one unit of DNase is defined for this figure as that amount rendering 100 pmol DNA-nucleotide acid-soluble.

found to be identical in this regard to the sucrose-gradient fraction (Table I). The electrophoretic migration of the ability to release oligonucleotides with *both* peaks of restriction endonuclease, coupled with the sedimentation data (Fig. 2) and reaction requirements (Table I) confirm that this activity is part of the restriction enzyme.

Characterization of the acid-soluble material: Paper electrophoresis at pH 3.5 shows that all counts migrate toward the anode with nucleotide material. (Bases and nucleosides would migrate toward the cathode). In addition, the same amount of acid-soluble material is noted with ^{32}P-labelled DNA as with [^{3}H]thymidine-labelled DNA. Therefore, the acid-soluble counts are in nucleotide form.

Chromatography of the acid-soluble material on paper in n-propanol-NH_4OH-H_2O (6:3:1), resulted in the radioactivity appearing in the regions of dinucleotides to octanucleotides and larger. No mononucleotides were observed. When the acid-soluble material was isolated from ^{32}P-labelled DNA, 14.5% of the ^{32}P could be converted to inorganic phosphate with bacterial alkaline phosphatase. By this criterion the average chain length of the acid-soluble product is thus 7. It should be noted that by virtue of our selection of acid-soluble products, we have excluded material larger than roughly 15 nucleotides in length (11). For this reason, the average chain length of small product may be larger, and indeed, more than 75 nucleotides may be released during a strand scission. Analysis of restricted DNA upon sucrose gradients gives no evidence for significantly more non-sedimentable material than we observe as acid-soluble, however.

^{32}P-labelled acid-soluble product was also digested to mononucleotides with pancreatic DNase and venom diesterase. Separation and quantitation of the mononucleotides showed no evidence for a predominance of any one of the four nucleotides; instead the base content appeared to reflect that of the substrate DNA.

Conclusions: The above results demonstrate that each single-stranded scission made by the *Eco* B restriction enzyme is accompanied by the release of roughly 75 nucleotides as oligonucleotides of average chain length 7 and random base composition. The time course of their formation (Fig. 1) shows that the release of oligonucleotides occurs during the early, DNase phase of the restriction reaction, not during the ATPase phase which continues for as long as several hours (4,5). A noteworthy question is whether the oligonucleotides are released as part of a single fragmentation event simultaneous with the endonucleolytic scission, or whether they represent the product of multiple events by a number of restriction enzyme molecules. The former situation has been observed with *E. coli* endonuclease I, which releases roughly 40 nucleotides upon cleaving DNA endonucleolytically (Wm. Studier, personal communication). On the other hand, Horiuchi, *et al.* (9) have suggested that once DNA is restricted to a minimal length by the *Eco* B enzyme, it is resistant to further degradation, even though the specificity sites are still intact. With DNA below minimal size they hypothesize that the enzyme molecules recognize the specificity site, traverse the length of the molecule, but "fall off" before cleaving the DNA. Perhaps in "falling off" the oligonucleotides that we have observed are released. If this were the case, the "fallen" enzyme would still be able to act upon another molecule, since we have observed that each restriction enzyme added to a reaction mixture ultimately cleaves DNA endonucleolytically before it dissociates into the ATPase state (1). Whatever the source of oligonucleotides, their presence must be accounted for in developing models for the complex *Eco* B restriction reaction.

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