A novel exocyttoplasmic endonuclease from *Streptomyces antibioticus*

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A new exocyttoplasmic, nutritionally controlled endodeoxyribonuclease (EC 3.1.21.-) was purified to homogeneity from *Streptomyces antibioticus*. The enzyme showed an apparent molecular mass of 29 kDa (being active in the monomeric form) and a pI of ~ 7.8 . The nuclease hydrolysed endonucleolytically double-stranded circular and linear DNA. The enzyme makes nicks in one strand of the DNA in G-rich regions, leaving either 5' or 3' short, single-stranded overhangs with 3'-hydroxy and 5'-phosphate termini. Breaks in the DNA occur when two nicks in opposite strands are close together. The enzyme had an optimum pH of 7.5 and an absolute requirement for bivalent cations and ≥ 100 mM NaCl in the reaction buffer. Activity was greatly

diminished in the presence of phosphate, Hg^{2+} or iodoacetate and was stimulated by dimethyl sulphoxide. Single-stranded DNA was a much poorer substrate than double-stranded DNA. The nuclease hydrolyses sequences of three or preferably more (dG)·(dC) tracts in the DNA. The initial specificity shifts to other sequences (including sequences shorter than those initially hydrolysed) during the course of the reaction, giving the changing pattern of bands observed in agarose gels. 5-Methylcytosine-hemimethylated DNA is not hydrolysed by the nuclease. The properties of this novel enzyme suggest a relationship with class II restriction endonucleases and also with some eukaryotic nucleases.

INTRODUCTION

Streptomyces species, along with many other bacterial species, are good producers of restriction enzymes [1]. As their life-cycle takes place in the soil, a place where they use a large variety of organic polymers, they are commonly considered to be excellent producers of hydrolytic enzymes. These include non-sequence-specific extracellular nucleases, which may perform a nutritional role, similar to that fulfilled by other degradative enzymes [2]. Very few studies have been carried out, in *Streptomyces*, to try to characterize DNAases other than restriction endonucleases [3,4]. We have made a survey of these enzymes in several species, which showed that all DNAases detected had endonucleolytic activity, similar molecular masses, required Mg^{2+} for optimal activity and were located in the cell-wall cytoplasmic membrane space, from where they were released after protoplast production [5]. Their synthesis was found to be dependent on the composition of the growth media (i.e., it was repressed by rich nitrogen sources, which promote high growth rates) and related to the stationary phase of growth [5]. These are the same conditions that trigger differentiation in *Streptomyces* [6]. One of these nucleases (the DNAase from *Streptomyces glaucescens*) was purified to homogeneity and characterized [7,8]. Other species (e.g. *Streptomyces antibioticus* ATCC11891) also produced a strong endonucleolytic activity assayed on DNA substrates; this activity gave rise, initially, to visible bands on agarose gels, which were subsequently degraded to shorter oligonucleotides [9]. This enzyme (as also occurs with the DNAase from *S. glaucescens* [10]), does not form part of a restriction-modification system but may fulfil a circumstantial role in phage restriction [9,11].

In this work we present the purification and biochemical characterization of the endonuclease from *S. antibioticus*. The enzyme cuts double-stranded and single-stranded linear and

circular DNA in G-rich regions, resulting, in the former case, in the production of double-stranded fragments with 5'-phosphate and/or 3'-hydroxy protruding single-stranded tails. The specificity alters during hydrolysis to other dinucleotides, giving the pattern of sequential DNA degradation characteristic of this enzyme. These properties could be related to the function postulated for this enzyme in the recycling of the nucleic acids components before the formation of aerial mycelium [12]. Apart from its hypothetical role in the cell, the remarkable characteristics of this previously undescribed nuclease makes this enzyme an interesting model to analyse and raises the question of its origin and evolutionary relationships with other DNA-degrading enzymes.

EXPERIMENTAL

Materials

pBR322 DNA, M13mp18 RF DNA, *Escherichia coli* tRNA, Tris base, 5-methyl-2'-deoxycytidine 5'-triphosphate (dm^5CTP), restriction endonucleases, calf intestine alkaline phosphatase (EC 3.1.3.1), T4 polynucleotide kinase (EC 2.7.1.78), T4 DNA ligase (EC 6.5.1.1), terminal deoxynucleotidyl transferase (TdT) (EC 2.7.7.31), Klenow enzyme, *E. coli* exonuclease III (EC 3.1.11.2), mung bean nuclease (EC 3.1.30.1), nuclease S1 (EC 3.1.30.1), micrococcal nuclease, and calf spleen phosphodiesterase (EC 3.1.16.1) were purchased from Boehringer-Mannheim; *E. coli* exonuclease I (EC 3.1.11.1) from United States Biochemical Corp. (Cleveland, OH, U.S.A.); Hepes, Mes, dAMP, dCMP, dGMP, dTMP; poly(dA-dT)·poly(dA-dT) and DNA-cellulose from Sigma Chemical Co.; poly(dG-dC)·poly(dG-dC) from P-L Biochemicals, Inc. (Milwaukee, WI, U.S.A.); silver staining reagents from Merck (Darmstadt, Germany); acrylamide and bisacrylamide from Serva (Heidelberg, Germany); SDS, am-

Abbreviations used: AdoMet, S-adenosyl-L-methionine; ddATP, 2',3'-dideoxyadenosine 5'-triphosphate; dm^5CTP , 5-methyl-2'-deoxycytidine 5'-triphosphate; DMSO, dimethylsulphoxide; DTT, 1,4-dithiothreitol; GAE, glucose, asparagine, yeast extract and salts; PMSF, phenylmethanesulphonyl fluoride; RF, replicative form; TdT, terminal deoxynucleotidyl transferase; TEMED, N,N,N',N'-tetramethylethylenediamine.

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monium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) from Bio-Rad Laboratories (Richmond, CA, U.S.A.); phosphocellulose P11 from Whatman Biosystems (Maidstone, Kent, U.K.); poly(dG)·poly(dC), Cibacron Blue F3-GA Sepharose, MonoS HR 5/5 f.p.l.c. column and the f.p.l.c. equipment from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden); and [γ - 32 P]ATP and 2',3'-[α - 32 P]-dideoxyadenosine 5'-triphosphate (ddATP) (3000 Ci/mmol) from Amersham (U.K.). All other chemicals used were from commercial sources and of reagent grade. Lambda single-stranded DNA was prepared by heating (100 °C, 5 min) double-stranded λ DNA (New England Biolabs, Inc., Beverly, MA, U.S.A.). Ribosomal RNA was isolated from *Streptomyces albus* G as described by Hopwood et al. [13]. Hemimethylated double-stranded DNA was synthesized by universal primer extension of M13mp18 single-stranded DNA, with dCTP replaced by dm⁵CTP as described [8].

Organisms and culture conditions

S. antibioticus ATCC11891 was used throughout this study. Sporulation on agar plates was achieved on GAE (glucose, asparagine, yeast extract and salts) medium at 30 °C [5]. Cells grown in the same liquid medium were used for enzyme purification [9].

Assay of endonuclease activity

Deoxyribonuclease activity was measured by a modified procedure based on that devised by Sadowski and Hurwitz [14] for T4 endonuclease IV. The basic reaction mixture (final volume 40 μ l) contained 10 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 7 mM 2-mercaptoethanol [or 1 mM 1,4-dithiothreitol (DTT)], 100 mM NaCl and 15 μ g of λ DNA [circularized with T4 polynucleotide ligase (Boehringer) as described in Hopwood et al. [13]]. After incubation for 30 min at 37 °C, the reaction was terminated by heating at 90 °C for 10 min. Water (49 μ l) was added and the DNA was denatured by boiling for 5 min. The sample was kept on ice for 10 min and then 10 μ l of I buffer (67 mM glycine, pH 9.5, 10 mM 2-mercaptoethanol and 6.7 mM MgCl₂) was added, followed by 1 unit of *E. coli* exonuclease I, sufficient to degrade all the free ends generated by the *Streptomyces* endonuclease. The reaction was then incubated for 10 min at 37 °C. After boiling for a further 5 min, trichloroacetic acid (final concentration 10% w/v) was added and the sample kept on ice for 15 min. The resulting precipitates were centrifuged and the A_{260} of the supernatant was determined. Endonuclease activity was expressed as the increase in the absorbance obtained under the reaction conditions described above or the conditions specified for each experiment. One unit of the enzyme activity is equivalent to the increase of 0.5 in the A_{260} after the treatment with exonuclease. K_m values were calculated by a non-linear regression analysis program (ENZFITTER, Elsevier-Biosoft). Activity in the fractions obtained from column chromatography was assayed by visualization of the products of DNA hydrolysis after gel electrophoresis. λ DNA (0.25 μ g) was incubated in 20 μ l of reaction buffer (see above) with a suitable volume of each column fraction (final NaCl concentration 0.08–0.12 M). Incubation was at 37 °C and the reactions were stopped after 1 h by the addition to each sample of 2.2 μ l of a stop solution [15]. The samples were loaded directly onto 0.8% (w/v) agarose gels. This method was also used to measure the ribonuclease activity of 2 μ g of the appropriate substrate.

Enzyme purification

All purification procedures were carried out at 4 °C unless otherwise stated. *S. antibioticus* mycelium (150 g wet weight) was washed and resuspended in 600 ml of buffer A [10 mM of potassium phosphate buffer, pH 7.0, 1 mM DTT, 1 mM EDTA, 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM Na₂S₂O₃] containing 0.2 M NaCl. Cells were broken with a French Pressure Cell Press (SLM & Aminco Instruments, Urbana, IL, U.S.A.) and further subjected to sonication at a 14 μ m amplitude for 30 s periods until complete rupture. The ruptured mycelium was ultracentrifuged at 40000 g for 1 h and the supernatant was then used as the source of the enzyme. The enzyme was purified from the supernatant as follows.

Step 1: Phosphocellulose chromatography

A phosphocellulose column was prepared according to Greene et al. [16]. The mycelium extract (970 mg of protein and 23 500 units of enzyme) was applied (flow rate 100 ml/h) to a phosphocellulose column (5.2 cm $\times$ 9.2 cm, 200 ml) equilibrated with buffer A plus 0.4 M NaCl. The column was washed with 1.0 l of this buffer before elution with a single solution of buffer A containing 0.8 M NaCl, from which $\sim$ 175 ml of active enzymatic solution was collected (31.5 mg of protein, 34 200 units).

Step 2: Affinity chromatography on Cibacron Blue F3-GA Sepharose

The peak of activity from the phosphocellulose column was diluted with 325 ml of 30 mM Tris/HCl, pH 8.0, 0.18 M NaCl; 7 mM 2-mercaptoethanol; 0.1 mM PMSF (to achieve a final concentration of 20 mM Tris HCl, 3.5 mM sodium phosphate; buffer B) and applied to a column of Cibacron Blue–Sepharose (3 cm $\times$ 13 cm, 20 ml), previously equilibrated with 200 ml of buffer B plus 0.4 M NaCl, at a flow rate of 29 ml/h. Proteins were eluted with a solution of buffer B plus 0.9 M NaCl.

Step 3: Affinity chromatography on DNA–cellulose

The active eluent ($\sim$ 100 ml) from the above column was dialysed against buffer B plus 0.1 M NaCl and applied to a double-stranded DNA–cellulose column (1 cm $\times$ 10 cm, 7 ml) previously equilibrated with the same buffer, at a flow rate of 9 ml/h. The enzyme was eluted with a 30 ml linear gradient of 0.1–1 M NaCl. The fractions containing enzyme activity eluted at $\sim$ 0.13 M NaCl. The final volume of pooled active fractions was 5.1 ml (8.2 mg of protein, 17 300 units).

Step 4: F.p.l.c. cation-exchange chromatography

The total active fractions from the former step were applied to a Mono S HR 5/5 column attached to an automated f.p.l.c. chromatographic system (Pharmacia), which had previously been equilibrated with 44 mM Hepes/KOH, pH 7.1, 7 mM 2-mercaptoethanol, 1 mM EDTA and 0.3 M NaCl at a flow rate of 1 ml/min. The enzyme was eluted with a 30 ml linear gradient of 0.3–1 M NaCl in the same buffer. The activity eluted in a single peak at 0.69 M NaCl. A total of 0.077 mg of pure protein (9230 units) was obtained. Pooled active fractions (containing $\sim$ 0.5 M NaCl) were stored at -20 °C in 44 mM Hepes/KOH, pH 7.1, 30% (v/v) glycerol and 10% (v/v) dimethylsulphoxide (DMSO).

Analytical methods

Protein concentrations were estimated using the Bradford method as modified by Spector [17], or by measuring the A_{280} of column eluates. The purity of the enzyme was assessed by

SDS/PAGE according to the method of Laemmli [18], using 3.9% (w/v) acrylamide in the stacking gels and 12% (w/v) acrylamide for the separating gels. Proteins were stained with silver nitrate according to the method of Morrissey [19]. For estimation of the molecular mass of the pure enzyme, SDS/PAGE analysis was performed as indicated above, including reference proteins as markers (Electrophoresis Calibration Kit, Pharmacia; 94–14.4 kDa). The native molecular mass of the nuclease was estimated by gel filtration using the f.p.l.c. system and a Superdex G-75 HR 10/30 column (Pharmacia) (0.3 ml/min flow) previously calibrated with proteins of known molecular mass (5–12 µg/sample; BSA, 66 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; cytochrome *c*, 12.3 kDa) in buffer B containing 0.5 M NaCl. The molecular mass and the purity of the enzyme preparation were also analysed by glycerol (10–30% w/v) gradient centrifugation as specified previously [5]. Fractions (175 µl) were assayed on λ DNA and the hydrolytic products were analysed by gel electrophoresis as described above. The isoelectric point of the nuclease was determined by chromatofocusing on a MonoP HR column using the f.p.l.c. system. Start buffer was 0.074 M Tris/ethanoic acid, pH 9.2, 10% (v/v) glycerol and elution buffer was Polybuffer 96 (Sigma)/ethanoic acid, pH 6.0, 10% (v/v) glycerol.

Size of DNA fragments generated by the *S. antibioticus* nuclease

Fragments generated by a total digestion of λ DNA were dephosphorylated with calf intestinal alkaline phosphatase and then labelled at the 5'-termini with T4 polynucleotide kinase and [γ -³²P]ATP, according to standard procedures [20,21]. Samples were heated and electrophoresed on polyacrylamide/urea gels [8]. The size distribution of fragments was determined by densitometric analysis of autoradiographs of the gel. pBR322 digested with *Hpa*II was used as a standard.

Detection of single-stranded tails

The production of DNA fragments with 5' protruding single-stranded tails was determined after a 3 h digestion of 0.5 µg of λ DNA, by labelling the fragments at their 5'-termini with polynucleotide kinase and [γ -³²P]ATP (see above), followed by treatment with single-strand specific endonucleases (mung bean nuclease or nuclease S1) [8]. The presence of 3' single-stranded overhangs was tested by labelling the fragments at their 3'-termini with 3' TdT and [α -³²P]ddATP in the presence of Co²⁺ [22], followed by treatment with exonuclease III [8]. In all cases, control reactions were performed simultaneously and consisted of λ DNA cut with *Eco*RI and *Hind*III (for 5' overhangs), λ DNA cut with *Pst*II/*Sac*I (for 3' overhangs) and λ DNA cut with *Hae*III (for blunt ends).

Determination of the phosphomonoester generated after cleavage of DNA

Phage λ DNA (0.4 µg) was partially digested with the endonuclease in 20 µl of reaction buffer (see above). Appropriate times of incubation and amounts of enzyme were determined by agarose gel electrophoresis of control samples. The reactions were terminated and DNA fragments purified by extraction with phenol/chloroform/isoamylalcohol (25:24:1, by vol.). Each sample was then divided into two aliquots. One was treated with calf intestine alkaline phosphatase (0.5 units) in 50 mM Tris/HCl, pH 8.0, 0.1 mM EDTA for 30 min at 37 °C. The reaction was stopped by the addition of 10 mM EGTA and by heating at 65 °C for 30 min, and the DNA was extracted with phenol/chloroform/isoamylalcohol. The other aliquot was left untreated.

Both samples were then labelled with [γ -³²P]ATP and T4 polynucleotide kinase as described above. To test the possibility of the production of 3'-hydroxy termini, 0.2 µg of λ DNA were digested with the nuclease and the DNA fragments generated were purified as described above. The samples were then labelled with [α -³²P]ddATP and TdT, as above. In all cases, non-incorporated [γ -³²P]ATP or [α -³²P]ddATP was separated by chromatography on Sephadex G-50 according to Sambrook et al. [15].

Analysis of the 5'-terminal nucleotide of endonuclease-generated fragments

This was carried out after digestion of λ DNA (2 µg) with the *S. antibioticus* endonuclease. DNA precipitation, dephosphorylation and labelling at the 5'-termini with T4 polynucleotide kinase and [γ -³²P]ATP were performed as described [8]. Analysis of the 5'-terminal nucleotide of the phosphorylated DNA was carried out after digestion with pancreatic DNAase I (EC 3.1.21.1) (10 µg) and snake venom phosphodiesterase (EC 3.1.15.1) (2 µg) for 2 h in 10 mM Tris/HCl, pH 7.5, 10 mM MgCl₂. Deoxyribonucleoside 5'-monophosphates were separated by t.l.c. using poly(ethyleneimine)cellulose F plates, as previously described [8].

Analysis of the 3'-terminal nucleotide

For the analysis of 3'-terminal nucleotide, DNA fragments generated by the nuclease (see above) were labelled at the 3'-termini using TdT and [α -³²P]ddATP, as already described. The ³²P label was transferred from ddAMP to the adjacent nucleotide by digestion to nucleoside 3'-monophosphates with micrococcal nuclease (10 µg) and calf spleen phosphodiesterase (10 µg) for 2 h at 37 °C in 10 mM Tris/HCl, pH 8.5, 10 mM MgCl₂, 5 mM CaCl₂. Details of these methods are given in a previous work [8]. These nucleoside 3'-monophosphates were separated as in the previous section, using nucleoside 3'-monophosphates as standards also as described previously [8].

Identification of the cleavage site of the nuclease

This was carried out by different approaches in DNAs of different G/C content. For cloned DNA substrates the method of Brown and Smith [23] was basically followed. M13mp18 single-stranded DNA with a 5'-phosphorylated universal sequencing primer was used for enzymatic sequencing [24] (Sequenase Version 2.0 DNA, USB, Cleveland, OH, U.S.A.) of the M13 DNA or of different DNA inserts. In parallel reactions, T4 polynucleotide kinase-labelled primer (see above) was extended by treatment with Klenow enzyme and all four dNTPs. This double-stranded DNA was used as substrate for the *S. antibioticus* DNAase to produce 5'-end-labelled DNA fragments comparable with the sequencing ladder. Another approach for identifying the cutting sites in DNA fragments was to compare the fragments produced by the nuclease with those produced by sequencing the same end-labelled DNA with base-specific chemical cleavages [25].

RESULTS

Purification of the *S. antibioticus* nuclease

The enzyme was purified from mycelium grown for 60 h (advanced stationary phase of growth) in GAE medium. No other major DNAase activity was detected throughout the process or using any of the analytical methods used during the study. A summary of the specific activity and recovery of the enzyme

Table 1 Purification of *S. antibioticus* nuclease

Pool	Protein (mg)	Endonuclease activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Extract	970	23 500	24	—	—
Phosphocellulose	31.5	34 200	1 085	145.5	45
Cibacron–Sephadex	17.6	22 700	1 289	96.6	53
DNA–cellulose	8.2	17 300	2 109	73.6	87
Mono S-f.p.l.c.	0.077	9 230	119 870	39.3	4994

Figure 1 SDS/PAGE of samples from the *S. antibioticus* nuclease purification

Samples from each step of the purification as described in the Results section were analysed by SDS/PAGE as described in the Experimental section. Lane A, molecular-mass markers (BSA, ovalbumin, carbonic anhydrase and trypsin inhibitor; numbers indicate the molecular masses in kDa); lane B, 4.3 units of phosphocellulose pool; lane C, 3 units of Cibacron–Sephadex pool; lane D, 2 units of DNA–cellulose pool; and lane E, 2.6 units of MonoS-f.p.l.c. pool.

during the purification procedure is given in Table 1. Each step of the purification was also analysed by SDS/PAGE followed by silver staining (Figure 1). The purified enzyme showed a single major protein band on SDS/PAGE. The enzyme was purified ~ 5000-fold with a yield of 39.27% from the cell extract. However, because of the increase of activity units on DNA observed after phosphocellulose chromatography (Table 1), the purification factor and the yield were actually lower. It is possible that the phosphocellulose step separated the enzyme from an inhibitor (such as phosphate and/or RNA; see below). This step also separated the enzyme from the nucleic acids present in the extracts, which may interfere with the assay. The increases in specific activity after recovery from the Cibacron Blue–Sephadex, the DNA–cellulose and the Mono S column were 1.18-, 1.63- and 57-fold respectively (Table 1). The purified enzyme rapidly lost activity, even in 30% (v/v) glycerol at –20 °C, unless 10% (v/v) DMSO was added (see below).

Molecular mass and isoelectric point

The molecular mass of the enzyme was estimated to be ~ 29 kDa by SDS/PAGE (Figure 1). Molecular-mass determination of the DNAase by gel filtration on Superdex 75 under non-denaturing conditions yielded a mass of ~ 28 kDa. Analysis by glycerol-gradient centrifugation resulted in a value of ~ 30 kDa, thus suggesting that the protein is a monomer. The *pI* of the *S. antibioticus* nuclease was found to be 7.8 ± 0.1 by f.p.l.c. chromatofocussing in Mono P.

Figure 2 Activity of the *S. antibioticus* nuclease on hemimethylated M13 DNA

Lane 1, M13 mp18 (single-stranded DNA) (0.4 µg); lane 2, M13mp18 (RF II form) (0.1 µg); lane 3, M13mp18 (RF II form) obtained by primer extension of single-stranded DNA of lane 1 (non-hemimethylated); lane 4, the same as lane 3, digested with 2.6 units of the *S. antibioticus* nuclease for 1 h; lane 5, the same as lane 3, digested with 10 units of *HhaI* (sensitive to DNA hemimethylated at cytosine) for 1 h; lane 6, the same as lane 3, digested with 6 units of *TaqI* (insensitive to DNA hemimethylated at cytosine) for 1 h; lanes 7–10, the same as lanes 3–6 but with hemimethylated DNA as substrate.

Substrate specificity and optimal conditions of the *Streptomyces* nuclease

The enzyme could hydrolyse native and denatured DNA. Hemimethylated DNA was not digested by the nuclease (Figure 2). Purified fractions exhibit some degradation of rRNA but not of tRNA. Additional studies are currently being carried out to elucidate whether this activity is associated with the nuclease or whether it is attributable to a residual contamination with a very active RNAase.

The pH dependence of enzyme activity on λ DNA was measured in 10 mM Mes/NaOH, pH 5.5–7.2, 10 mM Tris/HCl, pH 7.0–9.0, and 10 mM glycine/NaOH, pH 8.5–12.0 buffers. The enzyme showed maximum activity at pH 7.5. The optimal NaCl concentration was 100 mM. A 38% inhibition was observed at 120 mM NaCl, and KCl showed a similar effect. The optimum temperature of the enzyme was found to be in the range 37–50 °C. At 50 °C the initial activity was higher, although activity was lost rather more rapidly than at 37 °C, where the linear phase lasts much longer (results not shown). The stability of the enzyme was analysed at 50 °C for different times in the presence and absence of DMSO. The stability is noticeably higher (59% activity remains after 13 min of incubation, but only 28% remains in the absence of DMSO) in the presence of this organic solvent.

The analysis of the effect of divalent cations on DNAase activity showed that Mg^{2+} is required for activity; the optimal

Table 2 Effect of several compounds on nuclease activity

Nuclease assays (using 2.6 units of the purified enzyme) were carried out on λ DNA as described in the Experimental section. Complete system refers to the buffer normally used to measure the activity (10 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol or 1 mM DTT and 100 mM NaCl). Values in parentheses correspond to different concentrations of the assayed compound.

System	Activity (%)
Complete	100
– 2-Mercaptoethanol	12
+ Hg^{2+} (1, 5 and 10 mM)	82, 9, 0
+ Iodoacetate (1 and 10 mM)	14, 0
+ EDTA (5 and 10 mM)	16, 0
+ EGTA (10 mM)	10
+ DMSO (1, 5, 10 and 20%, v/v)	118, 145, 204, 315
+ Glycerol (5, 10 and 20%, v/v)	94, 87, 24
+ ATP (20 μ M, 200 μ M and 1 mM)	98, 98, 100
+ AdoMet (48, 96 and 192 μ M)	96, 100, 96
+ AdoMet (48 μ M), ATP (100 μ M)	100

concentration was 10 mM. Mn^{2+} could replace this cation efficiently (optimal concentration 5 mM), whereas Co^{2+} or Cu^{2+} at their optimal concentration (5 mM in both cases) only yielded 52 and 24 % respectively of the maximum activity attained with Mg^{2+} . The ions Ca^{2+} , Zn^{2+} , Ba^{2+} and Hg^{2+} could not substitute for Mg^{2+} . Ca^{2+} did not act synergistically with Mg^{2+} . Addition of up to 200 μ M *S*-adenosyl-methionine (AdoMet) or 1 mM ATP alone or mixed in the presence of Mg^{2+} (cofactors of type I or III restriction endonucleases) did not stimulate the activity (Table 2). Spermidine at 0.5–1 mM did not affect the activity, and concentrations of 5 mM or higher noticeably decreased it (results not shown). Glycerol (1–10 %) did not significantly affect the activity although higher concentrations were inhibitory. Other organic solvents such as 20 % (v/v) DMSO (a stabilizer for the enzyme; see above) stimulated the activity more than 2-fold (Table 2). The endonuclease activity was strongly reduced unless 1 mM DTT or 7 mM 2-mercaptoethanol was included in the reaction mix (Table 2). Additions of reagents such as 5 mM Hg^{2+} or 10 mM iodoacetate reduced strongly or abolished enzyme activity completely (Table 2). This suggests that a reduced form of the enzyme is required and that certain cysteine residues might be involved in DNA hydrolysis or in the structural stability of the enzyme.

Inhibitor sensitivity

Addition of chelating agents such as EDTA (5 mM) in the presence of 10 mM Mg^{2+} produced a remarkable inhibition of the activity. EGTA at the same concentration showed the same effect (Table 2). This further supports the requirement of divalent cations for the reaction. The addition of rRNA, but not tRNA, in excess to a reaction mixture inhibited the DNAase activity (results not shown). Addition of 5'-mononucleotides (dAMP, dCMP, dGMP and dTMP) inhibited DNAase activity at concentrations higher than 0.1 mM, whereas corresponding nucleosides (1 mM) had no effect (results not shown), thus suggesting that inhibition is caused by the phosphate residue. This supposition was reinforced when 15 mM sodium phosphate was used instead of 10 mM Tris/HCl (76 % inhibition), or when it was used as an inhibitor (87 % inhibition with 10 mM sodium phosphate buffer) in the standard reaction mixture.

Figure 3 Time course of *S. antibioticus* nuclease digestion of supercoiled DNA at low and high enzyme concentration

Reactions were carried out on pBR322 (1 μ g) at 37 °C with different diluted solutions of the purified enzyme. Lane 1, pBR322 alone; line 2, pBR322 digested with 10 units of *Bam*HI (linear form); lanes 3–6, pBR322 digested with 1 unit of nuclease for 30 s, 1 min, 2 min and 3 min respectively; lanes 7–9, pBR322 digested with 2 units of nuclease for 3, 5 and 10 min respectively; lanes 10–12, pBR322 digested with 10 units of nuclease for 2, 5 and 10 min respectively. OCL, open circular DNA forms; LF, linear forms; CCF, closed circular forms.

Kinetics of hydrolysis of DNA forms

The K_m of the enzyme for λ double-stranded DNA was 4.3 nM, whereas for single-stranded, denatured λ DNA it was 11.1 nM (2.68-fold greater). The kinetics of degradation of the supercoiled, circular and linear forms of pBR322 for short periods and at low and high enzyme concentration were analysed. As shown in Figure 3, the diluted enzyme initially produces nicks in circular supercoiled DNA, and gives open circular forms. After this, linear DNA was subsequently degraded with a characteristic pattern at high enzyme concentration. The size of DNA fragments generated from λ DNA by the nuclease ranged from 35 to 250 bp. The endonuclease did not generate acid-soluble material with native or single-stranded DNA as substrate, which suggests that the enzyme does not have an activity to expand nicks into small gaps. This was corroborated by using T4 DNA ligase, an enzyme that specifically repairs 5'-phosphate 3'-hydroxy nicks [26]. M13 replicative form I (RF_I) DNA was nicked with the endonuclease to convert most of the supercoiled form to open circular forms (RF_{II}). After heating for 10 min at 65 °C the sample was incubated with DNA ligase. Bands of covalently closed topoisomers were generated (results not shown), suggesting that the ligase acted on the nicks previously formed by the DNAase.

The linear forms produced by the enzyme from circular forms (and the fragments subsequently generated) can arise by the independent production of nicks in each strand of the helix. When these nicks are close to each other the stability of the helix is lost and the DNA breaks into fragments. In this case, it is conceivable that the number of nicks in each strand of the helix are in excess of the number of double-stranded breaks. Another possibility is that the products of the reaction result from true double-strand cleavage of the duplex (perhaps with a transient single-stranded intermediate). To test between these alternatives, a reaction was performed with the *S. antibioticus* DNAase and λ DNA under conditions that gave a full range of linear fragments. After the treatment of this sample with S1 nuclease, there was a clear shift to fragments of lower size, indicating the existence of numerous nicks in the fragments previously produced by the *S. antibioticus* nuclease. This suggests that the mechanism of double-

Figure 4 Early sites of endonuclease cleavage in pBR322

Reaction mixtures (lanes B–E) contained 0.25 µg of pBR322 DNA and nuclease buffer when appropriate, in a final volume of 20 µl. Lane A, λ DNA digested with *Pst*I; lane B, pBR322 alone; lane C, pBR322 digested with *Eco*RI (10 units) for 1 h; lane D, pBR322 digested with the *S. antibioticus* nuclease (0.1 units); lane E, pBR322 digested with the *S. antibioticus* nuclease and then with *Eco*RI. OCF, open circular DNA forms; LF, linear forms; CCF, closed circular forms.

strand break was the first of the two possibilities indicated above, i.e. through the production of nicks in the separate strands of the DNA.

If that mechanism of hydrolysis is found to be correct, then the ability of *S. antibioticus* endonuclease to nick double-stranded DNA implies the production of DNA fragments with either 5' or 3' short, single-stranded overhangs. Treatment of these fragments (after 5'-end-labelling) with mung bean nuclease or nuclease S1 produced a remarkable loss of radioactivity (92 and 95% respectively). Controls showed no equivalent loss on treating *Hae*III-cut λ DNA fragments. In the same way, the retention of radioactivity (25%) observed after treating 3'-end-labelled fragments with exonuclease III (41.7% of retention was observed in the *Pst*I/*Sac*I-digested DNA) indicated the presence of 3'-protruding single-stranded tails which are not accessible to this enzyme. These results confirm the presence of 5'- and 3'-stranded tails and corroborated the nicking mechanism for this nuclease.

Production of 3'-hydroxy and 5'-phosphate termini

The property of TdT to catalyse the addition of dNTPS to 3'-hydroxy ends of DNA fragments was used to identify the production of 3'-hydroxy termini by the *S. antibioticus* nuclease. The experiment was performed on λ DNA previously digested by the endonuclease to produce a full pattern of fragments (similar to that shown in Figure 3, lane 10). The incorporation of radioactivity in the presence of TdT (19.4%) indicated that the nuclease generated 3'-hydroxy termini in its cleavage of double-stranded DNA (a *Pst*I-λ digested DNA control produced 22.3% incorporation over the same period). The production of 5'-phosphate termini was indicated by the following experiment. λ DNA was digested by the endonuclease as above and the reaction stopped. The sample was then divided into two parts: one part was extensively treated with calf alkaline phosphatase and the other part was left untreated. The results showed that unless the products of the endonuclease cleavage were previously treated with alkaline phosphatase, the 5'-termini could not be efficiently labelled with [γ -³²P]ATP in the T4 polynucleotide kinase reaction (16.3% incorporation after 1 h). The low level of incorporated radioactivity (2.9%) in the alkaline-phosphatase-untreated sample is more likely to correspond to an exchange of 5'-phosphate terminal groups [21]. λ DNA digested with

Table 3 Analysis of terminal mononucleotides after endonuclease digestion of λ DNA

Reactions were performed as indicated in Table 2, at two different times: after 1 h (partial digestion) and overnight (end products). Determination of 3'- and 5'-terminal mononucleotides was carried out as described in the Experimental section. Control 1 was *Bam*HI-digested DNA; control 2 was *Eco*RI + *Bam*HI-digested DNA.

Labelled nucleotide	³² P incorporated into mononucleotide (% of total)			
	A	C	G	T
5'-Terminal (1 h digestion)	15	27	48	10
5'-Terminal (overnight)	13	34	41	12
3'-Terminal (1 h digestion)	9	9	78	4
3'-Terminal (overnight)	11	16	65	8
5'-Terminal (control 1)	7	8	79	6
3'-Terminal (control 2)	6	8	82	4

*Eco*RI/*Hind*III was used, in this case, as a control (18.5 and 4.1% incorporation respectively).

Early sites of endonuclease cleavage in pBR322

The kinetics of hydrolysis of closed circular DNA prompt the question as to whether the nicks produced by the *S. antibioticus* DNAase occur at a unique or at several specific sites. To assess this, a time-course reaction of pBR322 DNA with the endonuclease was carried out and the reaction was stopped when most of the nicked circular forms of DNA were converted to linear forms and no lower-molecular-mass intermediates were shown to have accumulated (Figure 4). Aliquots of these linear forms were then digested with *Eco*RI, which has only one single site within the pBR322 sequence, and the products were analysed by agarose gel electrophoresis. There were 15 bands visible below the full-size linear form, and these could be matched in eight pairs (assuming that a lower fragment of ~ 500 bp had been lost) to give full-size pBR322 molecules and so define eight sites of cleavage in the pBR322 DNA. From these findings and the results shown above it can be concluded that the *S. antibioticus* endonuclease makes specific cuts in its DNA substrate, cleaving pBR322 initially in several preferred regions.

Determination of the 3'- and 5'-terminal nucleotides of the DNA fragments

The nature of the 3'- and 5'-terminal nucleotides was determined after the hydrolysis of λ DNA for different times. Only two times (1 h and overnight) are represented in Table 3. The results did not differ significantly and showed a preference for dGMP in both 3'- and 5'-termini in the early and late stages of hydrolysis, although this preference was more pronounced for the 3'-termini than for the 5'-termini. Furthermore, the proportion of dCMP after a long reaction time was somewhat higher than after a short time. This nucleotide preference was also investigated by testing the cleavage of synthetic polynucleotides by the nuclease. Three were used: poly(dG-dC)·poly(dG-dC), poly(dG)·poly(dC), and poly(dA-dT)·poly(dA-dT). The results showed a preferential degradation of the first, a near total lack of degradation of the second and a total resistance to cleavage of the third. When S1

Figure 5 Sites of cleavage of DNA by the *S. antibioticus* endonuclease

(a) A fragment from the 5' region of the gene encoding the *Sa*I endonuclease [27] preceded by a short stretch of M13 polylinker was subjected to digestion with 2.6 units of the *S. antibioticus* endonuclease. The products were analysed in parallel with enzymatic sequencing ladders (see Experimental section). Lanes A, C, G and T, sequencing markers; lanes 1–4, the same DNA samples treated with the nuclease for 30, 60, 90 and 110 min respectively. (b) A portion of the sequence [indicated by the marks on the right-hand side of (a)] is shown. Major cleavage sites are indicated by large arrowheads. Minor cleavage sites are denoted by smaller arrowheads.

nuclease was added to the former digested substrates, a displacement of the bands was observed with poly(dG)·poly(dC) but not with poly(dA-dT)·poly(dA-dT). These data support the hypothesis, as already shown above using long DNA substrates, that the nuclease made nicks in the G-strand from the poly(dG)·poly(dC) but it did not significantly attack the C-strand.

Cleavage site of the nuclease

To localize precisely the sequences cut by the nuclease, DNAs from different origins were used as substrates and the cleavage site identified as detailed in the Experimental section. The nuclease has strong preference for regions rich in G, with a majority cut after the third base in five consecutive Gs (Figure 5).

Other sequences were also cut, and from densitometric analysis of a large number of cuts the following preference for the sequences hydrolysed could be established: $d(\text{GGG}\downarrow\text{GG}) > d(\text{GGG}\downarrow\text{GA}) = d(\text{GGG}\downarrow\text{GC}) = d(\text{GGG}\downarrow\text{GT}) > d(\text{GCG}\downarrow\text{GN})$, where $\downarrow$ indicates the cleavage point. Other types of sequences hydrolysed were: $d(\text{GGG}\downarrow\text{A}) = d(\text{GTG}\downarrow\text{G}) = d(\text{GAG}\downarrow\text{G}) > d(\text{GGG}\downarrow\text{T}) = d(\text{GCG}\downarrow\text{A}) > d(\text{GC}\downarrow\text{G}) = d(\text{GC}\downarrow\text{A}) = d(\text{GG}\downarrow\text{G}) = d(\text{GC}\downarrow\text{T}) = d(\text{GGT})$. These sequences were digested with less intensity when next to the preferred ones. In this case, the intensity of the hydrolysis increased in the course of the reaction. Finally, other minor cuts were identified, in a lesser proportion (~15% of the total) within the dinucleotides G/G, C/G and C/C.

Effect of DMSO and Mn^{2+} on the specificity of the hydrolysis

DMSO strongly potentiates the nuclease activity (Table 2). To see whether this effect was attributable to a loss of specificity (which could give rise to a kind of 'star' activity), DNA was hydrolysed by the nuclease at different times in the presence and in the absence of this agent and the sequences cut were analysed as indicated above. The results clearly showed that DMSO did not alter the specificity of the enzyme but did promote an

increase in the velocity of the reaction. Mn^{2+} , on the other hand, could substitute for Mg^{2+} as the cofactor in the hydrolysis. As shown in a similar experiment to that with DMSO, this cation did not change the specificity of hydrolysis of the nuclease (results not shown).

DISCUSSION

The enzyme purified from *S. antibioticus* (EC 3.1.30.-) corresponds to the nuclease whose location, nutritional regulation of production and possible functions have been previously analysed [9,11,12]. Some of its properties resemble those of the DNAase previously purified from *S. glaucescens* [7], as both of them require Mg^{2+} and produce nicks in double-stranded DNA. However, differences were encountered in the molecular mass (39.6 and 29.0 kDa for the *S. glaucescens* and the *S. antibioticus* enzyme respectively), requirements for NaCl (a high concentration is necessary for the *S. antibioticus* enzyme but not for the other), stabilizing effect of DMSO and, above all, in the specificity of substrate and hydrolysis shown by both enzymes [8] (see below). Whereas the nuclease from *S. glaucescens* showed an apparently unspecific pattern of DNA degradation, as judged from the appearance of a continuous smear of hydrolytic products on agarose gels [10], the enzyme from *S. antibioticus* degraded DNA substrates producing, initially, specific bands on agarose gels, which were gradually hydrolysed to shorter segments in a continuous fashion (Figure 3). It was initially thought that this could be caused by the presence of a type II restriction endonuclease contaminated by some other DNAase. The purification and analysis of the enzyme, described in this work, eliminates this possibility. In addition, no modification enzyme was detected in the cell which could conceivably protect the host DNA from the nuclease [9].

The experiments performed with the nuclease-induced linear forms and *Eco*RI on pBR322 show that the *S. antibioticus* endonuclease makes specific cuts in its DNA substrates, cleaving pBR322 initially in about eight preferred regions. This experiment and the observable pattern of bands produced on agarose gels by the enzyme in the course of the hydrolysis (Figure 3) strongly suggested that the sequence specificity of the DNAase must be greater than just two nucleotides. Also, the t.l.c. analysis of 5'- and 3'-terminal nucleotides (Table 3), and the appearance of the different band patterns in the gels right up to the end products, implies that the initial specificity of the *S. antibioticus* DNAase possibly changes to other 'less canonical' sequences during the course of the reaction. A further analysis of the sequences cut by the DNAase showed that in fact the enzyme has a strong preference for sequences of three or more (dG)·(dC) tracts in the DNA and cuts preferentially after the third G.

Some of the properties shown by this enzyme would support the proposed function for the nuclease in the recycling of nucleic acids from the substrate mycelium [12]. These include the hydrolytic specificity, as *Streptomyces* DNA has a high G+C content. However, as the hydrolytic products which originated this nuclease on the DNA are of a relative high size, these could not be re-used directly for subsequent DNA synthesis unless they were degraded again by other nuclease. This assumption has recently been reinforced, as we have found a second non-specific DNAase produced in surface cultures (which have undergone aerial mycelium production) but not in submerged, non-differentiating cultures of *S. antibioticus* (the conditions used to purify the nuclease) (R. Nicieza, S. Cal and J. Sánchez, unpublished work).

The fact that the nuclease from *S. antibioticus* is a monomer and showed a strong sequence preference suggests some relation-

ships with the class II restriction enzymes. The enzyme, on the other hand, shows punctual analogies with some eukaryotic nucleases, for example, with a mammalian endonuclease which shows site-specific cleavage of DNA [28]. The common aspects include requirements for Mg^{2+} , stimulation by DMSO, production of 3'-hydroxy and 5'-phosphate termini and the production of a specific band pattern following DNA hydrolysis, plus a progressive degradation of the intermediate forms to end fragments with a modal value of 120 bp. The differences include a true double-stranded cleavage of the DNA produced by the mammalian endonuclease (although there may be a transient and specific single-stranded intermediate in the reaction), and less specificity of this enzyme with respect to the sequences cut: guanine and adenine were preferably detected as the 5'-terminal nucleotide (3'-terminal nucleotides were not analysed) and poly(dA)·poly(dT), poly(dA·dT) and poly(dG)·poly(dC) were hydrolysed with effectiveness analogous to other DNA substrates. A nuclease which produced specific, high-molecular-mass bands on agarose gels has also been reported in the eukaryote alga *Chlamydomonas* [29]. This enzyme produces nicks and gaps in one strand of DNA and when two of them on complementary strands coincide (as occurs in *S. antibioticus*) double-stranded breaks are produced. Thymidylic acid, followed by deoxyguanylic acid, were the predominant 5'-terminal nucleotides after cleavage of DNA. Thus, none of these nucleases has the specificity shown by the enzyme from *S. antibioticus*.

More similar to the enzyme of *S. antibioticus* could be the endonuclease G, a (dG)_n·(dC)_n-specific DNAase-RNAase from vertebrates which has been related with mitochondrial DNA replication [30,31]. Also similar to endonuclease G are the DNAases located in the human heart, bovine heart and rat heart mitochondria [32–34]. In the absence of the (dG)_n·(dC)_n sequences, the cuts produced by these enzymes in the DNA substrates resemble in their disposition those produced by the *S. antibioticus* nuclease [35]. The kinetics of hydrolysis of the endonuclease G is highly specific for (dG)_n·(dC)_n [30]. However, this enzyme, in contrast to *S. antibioticus* DNAase, nicks the C-strand with similar effectiveness to that shown in G-strand [31]. It will be interesting to learn whether the enzyme from *S. antibioticus*, in common with endonuclease G, shows high preference for regions with long (dG)·(dC) tracts (i.e., 18 bp or more) which adopt non-Watson-Crick DNA structures when they are present in supercoiled DNA [35]. Some experiments already performed show that the hydrolytic pattern produced by both endonuclease G and the nuclease from *S. antibioticus* on a plasmid with a (dG)₁₇·(dC)₁₇ insert [36] are different (A. Ruiz-Carrillo, personal communication).

The enzyme from *S. antibioticus* is a novel nuclease which should prove useful as a new tool in DNA analysis and as a model to analyse DNA-nuclease interaction. Cloning of the nuclease gene will help to elucidate the existence of regions similar to other nuclease genes and clarify the origin of the enzyme and its evolutionary relationships.

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