Purification and characterization of a nutritionally controlled endodeoxyribonuclease from *Streptomyces glaucescens*

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Streptomyces glaucescens has a DNAase whose synthesis is under nutritional control. We have purified this enzyme to apparent homogeneity by phosphocellulose chromatography followed by heparin-agarose, Cibacron Blue F3-GA-Sephadex and Sephadex G-75 chromatography and MonoQ f.p.l.c. The enzyme had an apparent M_r of 39 600 and a pI of approx. 8.15. The M_r of the native enzyme estimated by gel chromatography was 49 000. The DNAase had a pH optimum of 7.5 and an absolute requirement for bivalent cations in the reaction buffer. It was inhibited by high salt concentrations, chelating agents or phosphate-containing compounds and was stimulated by dimethyl sulphoxide. The activity was greatly diminished unless dithiothreitol or 2-mercaptoethanol was included in the reaction mixture. Reagents such as Hg^{2+} or iodoacetate strongly inhibited the enzyme. The nuclease hydrolysed both double-stranded and single-stranded DNA, showing greater affinity for double-stranded DNA, and no detectable hydrolysis of RNA. The enzyme produced nicks in double-stranded DNA, generating 3'-hydroxy and 5'-phosphate termini, and degraded circular DNA.

INTRODUCTION

Streptomyces species are excellent producers of hydrolytic enzymes, since their life-cycle takes place in the soil, where they use a great variety of organic polymers [1]. Thus sequence-non-specific extracellular nucleases would be likely to have a nutritional role similar to other enzymes such as cellulases, amylases, xylanases, lipases and proteinases. In addition to this hypothetical nutritional role, the possible implications of these DNAases for other processes such as recombination, conjugation and transformation and for DNA replication and repair cannot be ignored [2,3]; the DNAase activity of *Streptomyces glaucescens* has been implicated in phage restriction [4].

The *S. glaucescens* enzyme is a unique sequence-non-specific deoxyriboendonuclease located outside the cellular cytoplasm in the cell-wall/membrane region (J. F. Aparicio, S. Cal, J. F. Santos, C. Hardisson & J. Sánchez, unpublished work), whose synthesis is under nutritional control (i.e. it is repressed by rich nitrogen sources, which promote high growth rates) and related to the early stationary phase of growth [4], the same conditions that trigger differentiation in *Streptomyces* [5].

This striking repression of activity in response to clearly defined and easily manipulated nutrient signals justifies the further study of the *S. glaucescens* enzyme as a model regulatory system in *Streptomyces*. To understand the molecular basis of these phenomena it is essential to purify the enzyme to homogeneity. In the present study the periplasmic endodeoxyribonuclease of *S. glaucescens* was purified, and some of the physicochemical and enzymic properties of this enzyme were investigated.

EXPERIMENTAL

Materials

Tris base, calf intestine alkaline phosphatase, bacteriophage T4 polynucleotide kinase and terminal deoxynucleotidyltransferase were purchased from Boehringer Mannheim (Mannheim, Germany), Mes, Sephadex G-50, *Escherichia coli* tRNA and

heparin-agarose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), silver staining reagents were from Merck (Darmstadt, Germany), acrylamide and bisacrylamide were from Serva (Heidelberg, Germany), SDS, ammonium persulphate and *NNN'*-tetramethylethylenediamine were from Bio-Rad Laboratories (Richmond, CA, U.S.A.), nutrient broth was from Oxoid (Basingstoke, Hants., U.K.), phosphocellulose P11 was from Whatman Biosystems (Maidstone, Kent, U.K.), Sephadex G-75, Cibacron Blue F3-GA-Sephadex, MonoQ HR 5/5 f.p.l.c. column, low-range M_r standards for SDS/PAGE, wide-range pI standards, marker proteins for M_r determination by gel filtration and pH 3–10 ampholytes were from Pharmacia LKB Biotechnology (Uppsala, Sweden), Aquacide II was from Calbiochem (La Jolla, CA, U.S.A.), [γ - ^{32}P]ATP (3000 Ci/mmol) was from NEN Research Products (Boston, MA, U.S.A.) and [α - ^{32}P]ddATP (3000 Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.). All other chemicals used were from commercial sources and were of reagent grade. Single-stranded bacteriophage λ DNA was prepared by heating (100 °C for 5 min) double-stranded bacteriophage λ DNA. rRNA was isolated from *Streptomyces* 70 S ribosomal subunits obtained as described by Quirós *et al.* [6].

Organisms and culture conditions

S. glaucescens ETHZ 22794 was used throughout this study. Sporulation on agar plates was achieved on GAE medium at 30 °C [7]. Growth for enzyme purification was achieved on Oxoid nutrient broth supplemented with 0.5% (w/v) glucose (NBG medium).

Determination of protein concentrations

Protein concentrations were determined by using the Bradford [8] method, as modified by Spector [9], with BSA as standard, or by measuring the absorbance of column eluates at 280 nm.

Assay of endonuclease activity

Endonuclease activity was normally measured by the formation of acid-soluble DNA fragments. The standard reaction mixture contained 10 mM-Tris/HCl buffer, pH 7.5, 20 mM-NaCl,

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1 mM-dithiothreitol, 10 mM-MgCl₂, 20 nM bacteriophage λ DNA, and an appropriate amount of endonuclease in a total volume of 50 μ l. After incubation at 37 °C for 10 min, the reaction was terminated by the addition of calf thymus DNA (0.5 μ g/ μ l) as a carrier and 60 μ l of ice-cold 20 % (w/v) trichloroacetic acid. After the sample had been kept on ice for 15 min, the resulting precipitates were centrifuged down, and the absorbance at 260 nm of the supernatant was determined. Endonuclease activity was expressed by the increase in the absorbance obtained under the reaction conditions described above or the conditions specified for each experiment. In some experiments various components in the reaction mixture and the reaction time were changed as indicated. This assay was used throughout the present study unless otherwise indicated. One unit of the enzyme activity is equivalent to an increase in A_{260} of 0.02 obtained under the above reaction conditions.

Activity in the fractions obtained from column chromatography was assayed by measurement of the products of DNA hydrolysis after gel electrophoresis. The basic reaction buffers contained 10 mM-Tris/HCl buffer, pH 7.5, 10 mM-MgCl₂ and 1 mM-dithiothreitol. Bacteriophage λ DNA (0.5 μ g) was incubated in 30 μ l of reaction buffer with 2 μ l of each column fraction. Incubation was at 37 °C, and after 1 h the reactions were stopped by the addition to each sample of 3.3 μ l of a stop solution (containing glycerol, EDTA, SDS and Bromophenol Blue [10]). The samples were loaded directly on to 0.8 % agarose horizontal gels.

Gel electrophoresis and staining

SDS/PAGE was conducted according to the method of Laemmli [11], with 3.9 % acrylamide in the stacking gels and 12 % acrylamide for the separating gels. Protein titration curves were obtained by carrying out non-denaturing PAGE (5 % acrylamide) of proteins uniformly applied across a pH gradient, which was previously formed with pH 3–10 ampholytes by isoelectric focusing. The catholyte was 0.1 M-H₂SO₄ and the anolyte was 0.2 M-ethylenediamine. Electrophoresis was performed on an FBE 3000 flat-bed apparatus (Pharmacia LKB Biotechnology), and fixation of proteins was carried out with a 5 % (w/v) sulphosalicylic acid/10 % (w/v) trichloroacetic acid mixture during 60 min. The pI positions of the first-dimensional electrophoresis were determined with pI standards. These were trypsinogen (pI 9.3), lentil lectin (pI 8.65, 8.45 and 8.15), myoglobin (pI 7.35 and 6.8), carbonic anhydrase (pI 5.9), β -lactoglobulin A (pI 5.1) and trypsin inhibitor (pI 4.6).

Proteins were stained with AgNO₃ according to the method of Morrissey [12].

Determination of the phosphomonoester generated after cleavage of DNA

A 400 ng portion of bacteriophage λ DNA was partially digested with endonuclease in 20 μ l 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 were purified by extraction with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). Each sample was then divided into two aliquots: one was treated with calf intestine alkaline phosphatase (0.5 unit) in 50 mM-Tris/HCl buffer, pH 8, containing 0.1 mM-EDTA for 30 min at 37 °C (the reaction was stopped by addition of 10 mM-EGTA and heating at 65 °C for 30 min, after which an extraction was carried out with phenol/chloroform/3-methylbutan-1-ol), and the other part was left untreated. All samples were then labelled with [γ -³²P]ATP and bacteriophage

T4 polynucleotide kinase according to standard procedures [13,14].

To test the possibility of the production of 3'-hydroxy termini, 200 ng of bacteriophage λ DNA was digested with the nuclease and the DNA fragments generated were purified as indicated above. The samples were then labelled with [α -³²P]ddATP and terminal deoxynucleotidyltransferase in the presence of Co²⁺ [15].

In all cases non-incorporated [γ -³²P]ATP and [α -³²P]ddATP were separated by chromatography on Sephadex G-50 according to the procedure of Sambrook *et al.* [16].

Enzyme purification

All purification procedures were carried out at 4 °C unless otherwise stated. *S. glaucescens* mycelium (130 g wet wt.) was washed and resuspended in 600 ml of a solution containing 10 mM-potassium phosphate buffer, pH 7.0, 1 mM-dithiothreitol, 1 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride and 1 mM-Na₂S₂O₃ (buffer A) and also 0.2 M-NaCl. The suspension was sonicated at 14 μ m frequency for 30 s periods, until complete rupture, and the ruptured mycelium was ultracentrifuged at 40000 g for 1 h. Supernatant was then used as the source of enzyme.

Phosphocellulose chromatography. The phosphocellulose column was prepared according to the procedure of Greene *et al.* [17]. The former enzyme extract (1183 mg of protein and 4550 units of enzyme) was applied (flow rate 118 ml/h) to a phosphocellulose column (5.2 cm $\times$ 9.2 cm; 200 ml) equilibrated with buffer A containing 0.2 M-NaCl. The column was washed with 1 litre of this buffer before elution with a 1400 ml linear gradient of 0.2–0.8 M-NaCl in buffer A. Fractions (10 ml) were collected and assayed for activity. Active fractions were combined and designated as the phosphocellulose pool (260 ml).

Heparin-agarose chromatography. The phosphocellulose pool (37.5 mg of protein and 6592 units of enzyme) was diluted 1:4:5 in a solution containing 24 mM-Tris/HCl buffer, pH 8.0, 1 mM-dithiothreitol, 1 mM-EDTA and 0.1 mM-phenylmethanesulphonyl fluoride and applied (flow rate 30 ml/h) to a heparin-agarose column (2.2 cm $\times$ 4.7 cm; 17 ml) equilibrated with a solution containing 20 mM-Tris/HCl buffer, pH 8.0, 1 mM-dithiothreitol, 1 mM-EDTA and 0.1 mM-phenylmethanesulphonyl fluoride (buffer B) and also 0.1 M-NaCl. The column was washed with 70 ml of this buffer before elution with a 120 ml linear gradient of 0.1–1 M-NaCl in buffer B. Fractions (1.5 ml) were collected and assayed for activity.

Cibacron Blue F3-GA-Sepharose chromatography. The heparin-agarose pool (50 ml containing 12.4 mg of protein and 4368 units of enzyme) was diluted 3-fold in buffer B and applied (flow rate 20 ml/h) to a Cibacron Blue F3-GA-Sepharose column (2.1 cm $\times$ 4.5 cm; 15 ml) equilibrated with buffer B containing 0.2 M-NaCl. The column was washed with 40 ml of this buffer and the enzyme was eluted with a 100 ml linear gradient of 0.2–1 M-NaCl in buffer B. Fractions (1 ml) were collected and assayed for activity. Active fractions (42 ml) were combined, concentrated to 2 ml by dialysis against dry CM-cellulose (Aqua-cide II) and designated as the Cibacron-Sepharose pool.

Sephadex G-75 chromatography. The Cibacron-Sepharose pool (1.23 mg of protein and 3873 units of enzyme) was applied (flow rate 8 ml/h) to a Sephadex G-75 column (2 cm $\times$ 40 cm; 125 ml) equilibrated with buffer B containing 0.5 M-NaCl. Fractions (2 ml) were collected and assayed for activity. The M_r of the enzyme was calculated from the elution constant (K_{av} , [18]) compared with those of the standard proteins BSA (M_r 67000), ovalbumin (M_r 43000), chymotrypsinogen A (M_r 25000) and RNAase (M_r 13700).

MonoQ f.p.l.c. The Sephadex G-75 pool (0.86 mg of protein

Table 1. Purification of *S. glaucescens* nuclease

The nuclease from *S. glaucescens* was purified and assayed for protein concentration and activity as described in the Experimental section.

Step	Protein (mg)	Endonuclease activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Extract	1183	4550	3.84	100	1
Phosphocellulose pool	37.5	6592	176	144.9	46
Heparin-agarose pool	12.4	4368	352	96.0	92
Cibacron-Sephadex pool	1.23	3873	3149	85.1	820
Sephadex G-75 pool	0.86	3349	3894	73.6	1014
MonoQ pool	0.03	1150	38333	25.2	9983

and 3349 units of enzyme) was concentrated to 2 ml in a Centricon 10 microconcentrator (Amicon), diluted 20-fold in 50 mM-Tris/HCl buffer, pH 8.9, and again concentrated to 2 ml, and applied (flow rate 1 ml/min) to a MonoQ HR 5/5 column equilibrated in 50 mM-Tris/HCl buffer, pH 8.9. The column was washed with 4 ml of this buffer and the enzyme was eluted with a 12 ml gradient of 0–0.3 M-NaCl in 50 mM-Tris/HCl buffer, pH 8.9, followed by a 3 ml gradient of 0.3–1 M-NaCl in the same buffer. The enzyme solution was concentrated in a Centricon 10 microconcentrator, dialysed against a solution containing 10 mM-Tris/HCl buffer, pH 7.5, 0.2 M-NaCl, 0.1 mM-EDTA, 1 mM-dithiothreitol and 50% (v/v) glycerol (storage buffer), and preserved at -20°C .

RESULTS

Purification of enzyme

The *S. glaucescens* DNAase was purified from mycelium grown for 64 h (advanced stationary phase of growth) in NBG

Fig. 1. Titration curves of proteins from the Sephadex G-75 pool

Gel was previously focused with pH 3–10 ampholytes by isoelectric focusing as described in the Experimental section. The asterisk (*) denotes the titration curve of purified enzyme, which was identified as the result of an independent experiment with purified nuclease. pI standards were: T, trypsinogen (pI 9.3); LL, lentil lectin (pI 8.65, 8.45 and 8.15); MG, myoglobin (pI 7.35 and 6.8); CA, carbonic anhydrase (pI 6.9); L, β -lactoglobulin A (pI 5.1); TI, trypsin inhibitor (pI 4.6).

Fig. 2. SDS/PAGE of samples from the *S. glaucescens* 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. The amounts of enzyme applied were: lane A, 0.2 unit of phosphocellulose pool; lane B, 0.4 unit of heparin-agarose pool; lane C, 6 units of Cibacron-Sephadex pool; lane D, 6 units of Sephadex G-75 pool; lane E, 30 units of MonoQ pool.

medium by using the purification procedures described in the Experimental section. No other major DNAase activity was detected throughout the process.

Phosphocellulose chromatography. During chromatography on phosphocellulose the enzyme was eluted over a broad range of salt gradient with a peak elution at 0.57 M-NaCl. The specific activity of the pooled active fractions was 45-fold greater than that of the extract. Possibly this step separated the enzyme from an inhibitor substance present within the extracts that was not adsorbed on the column, since nuclease activity units of the pooled active fractions were 1.45-fold greater than those of the extract. As this step separated the enzyme from the nucleic acids of the extracts, and tRNA inhibits activity (see below), a feasible explanation for this increase in activity is the removal of tRNA.

Affinity chromatography. The phosphocellulose pool was chromatographed on heparin-agarose; the enzyme was eluted as a broad peak at 630 mM-NaCl. The pooled active fractions, which showed a 2-fold increase in specific activity and which contained 66% of the applied nuclease activity (Table 1), were loaded on to a Cibacron Blue F3-GA-Sephadex column. The

enzyme was eluted at 0.6 M-NaCl. The Cibacron-Sephadex pool (see the Experimental section) exhibited a 9-fold increase in specific activity with an 88 % yield of nuclease activity.

Sephadex G-75 chromatography and native M_r . The Cibacron-Sephadex pool was chromatographed on Sephadex G-75, and the enzyme elution volume corresponded to that of a globular protein of M_r 49000 (results not shown). The pooled active fractions contained 86 % of the DNAase activity loaded on to the column, and showed a 1.23-fold increase in specific activity.

MonoQ f.p.l.c. and titration curves. In order to know the range of pH that we had to use for separating the remaining proteins of the Sephadex G-75 pool, we obtained titration curves as described in the Experimental section. The pH turned out to be greater than 8.5 (Fig. 1). As enzyme activity decreased at pH values greater than 9.5 (see below), we chose a pH 8.9 to develop MonoQ f.p.l.c. The enzyme activity was eluted as a sharp peak at 150 mM-NaCl, which contained nearly 35 % of the applied DNAase activity (Table 1) and exhibited a 9.8-fold increase in specific activity.

Purification summary. The yields and specific activities of the enzyme during each step of the purification are shown in Table 1. Each step of the purification was also analysed by SDS/PAGE followed by silver staining (Fig. 2).

The DNAase of *S. glaucescens* was purified 9983-fold with a yield of 25 % from the cell extract. However, because of the increase of activity units observed after phosphocellulose chromatography (Table 1), the purification factor and the yield were actually lower.

Stability of purified enzyme. The purified enzyme was fairly stable in storage buffer. No appreciable loss of enzyme activity was detected during storage at -20°C for at least 16 months. Repeated freezing and thawing, however, resulted in a considerable loss of enzyme activity.

M_r and isoelectric point

The gel pattern of the MonoQ pool after SDS/PAGE revealed a single silver-stained band at M_r 39600 $\pm$ 700 (Fig. 2). The

Fig. 3. Effects of bivalent cations on nuclease activity

The activity of 3 nM enzyme was measured as described in the Experimental section except that the concentrations of bivalent cations ($\circ$, Mg^{2+} ; $\bullet$, Mn^{2+} ; $\triangle$, Co^{2+} ; $\blacktriangle$, Ca^{2+}) were changed as indicated. Activities were compared with that of the enzyme incubated with 10 mM- MgCl_2 in the reaction mixture.

titration curve of this protein (see the Experimental section) (Fig. 1) revealed a pI of approx. 8.15.

Effects of bivalent cations and other cofactors

The effects of bivalent cations on nuclease activity are shown in Fig. 3. The results showed the absolute necessity of Mg^{2+} for activity. The optimal concentration was 10 mM. Mn^{2+} could replace this cation efficiently (optimal concentration was 5 mM; Fig. 3), but Co^{2+} and Ca^{2+} yielded only 66 % and 15 % respectively of the maximum activity attained with Mg^{2+} (optimal concentrations were 10 mM and 15 mM in each case). Cu^{2+} , Zn^{2+} ,

Table 2. Effects of different reagents on nuclease activity

Nuclease assays were carried out on bacteriophage λ DNA as described in the Experimental section. Reaction mixtures contained approx. 3 nM enzyme.

System	Activity (%)	System	Activity (%)
Original buffer	100	- Mg^{2+}	0
- 2-Mercaptoethanol	50	- Dithiothreitol	48
+ Spermidine (1-10 mM)	100	+ SDS (100 μM)	0
+ Triton X-100 (0.1 %)	99	+ Urea (10 mM)	100
+ Formamide (100 μM)	85	+ Formamide (1 mM)	60
+ Formamide (10 mM)	25	+ EDTA (100 μM)	127
+ EDTA (1 mM)	37	+ EDTA (10 mM)	0
+ EGTA (1 mM)	108	+ EGTA (10 mM)	25
+ Hg^{2+} (10 μM)	92	+ Hg^{2+} (100 μM)	0
+ Iodoacetate (10 μM)	98	+ Iodoacetate (100 μM)	75
+ Iodoacetate (1 mM)	0	+ BSA (100 $\mu\text{g/ml}$)	180
+ Dimethyl sulphoxide (1 %)	120	+ Dimethyl sulphoxide (5 %)	132
+ Dimethyl sulphoxide (10 %)	235	+ Dimethyl sulphoxide (20 %)	350
+ Glycerol (1-20 %)	100-90	+ Glycerol (40 %)	20
+ ATP (30 μM)	100	+ ATP (200 μM)	48
+ Phosphate buffer (100 μM)	98	+ Phosphate buffer (1 mM)	75
+ Phosphate buffer (10 mM)	38	+ Phosphate buffer (0.1 M)	14
+ dAMP, dCMP, dGMP, dTMP (10 μM)	96, 93, 95, 89	+ dAMP, dCMP, dGMP, dTMP (100 μM)	52, 55, 42, 46
+ dAMP, dCMP, dGMP, dTMP (1 mM)	42, 45, 36, 40	+ dAMP, dCMP, dGMP, dTMP (10 mM)	24, 26, 0, 12
+ dAdo, dCyd, dGuo, dThd (1 mM)	95, 90, 92, 92	+ tRNA (3 μM)	66
Phosphate buffer (15 mM; pH 7.5)	18	Phosphate buffer (0.1 M; pH 7.5)	6

Fig. 4. Effect of salt concentration on nuclease activity

The activity of purified *S. glaucescens* nuclease (3 nM) was measured as described in the Experimental section except that the concentration of NaCl (○) or KCl (●) was varied as indicated. Activities were compared with that of the enzyme incubated with 20 mM-NaCl in the reaction buffer.

Fig. 5. Effect of pH on nuclease activity

The activity of the purified enzyme was assayed as described in the Experimental section except that the buffer in the reaction mixture was replaced by the following buffers with the indicated pH: 10 mM-Mes/NaOH buffer, pH 5.5–7.2 (○); 10 mM-Tris/HCl buffer, pH 7.0–9.0 (●); 10 mM-glycine/NaOH buffer, pH 8.5–12 (△). The enzyme activity obtained under the standard assay conditions (see the Experimental section) was set at 100%.

Ba²⁺ and Hg²⁺ could not substitute for Mg²⁺. Ca²⁺ did not act synergistically with the Mg²⁺. Addition of 30 μM-S-adenosyl-methionine or 30 μM-ATP in the presence of Mg²⁺ (cofactors of type I or III restriction endonucleases) did not stimulate the activity, although there was a 50% inhibition of activity with 200 μM-ATP (Table 2). Addition of 1–10 mM-spermidine did not affect the activity, even at low Mg²⁺ concentration (1 mM). The addition of dimethyl sulfoxide stimulated the nuclease activity

in the presence of Mg²⁺, with a maximal stimulation of 3.5-fold at a concentration of 20% (v/v). One possible explanation is that dimethyl sulfoxide might stimulate the activity by lowering the dielectric constant of the reaction mixture; however, other organic solvents that would also be expected to lower the dielectric constant, such as glycerol (1–20%, v/v), were not stimulatory. The addition of BSA (100 μg/ml) also stimulated activity (Table 2).

Effects of NaCl and pH

The optimal NaCl concentration was 20 mM. The enzyme was inhibited by higher concentrations (Fig. 4), and a 60% inhibition was observed at 100 mM-NaCl. KCl showed a similar effect (Fig. 4).

The pH optimum for the endonuclease activity was assessed by cleavage of bacteriophage λ DNA in the pH range 5.5–12. Three buffer systems were used, 10 mM-Mes/NaOH, pH 5.5–7.2, 10 mM-Tris/HCl, pH 7.0–9.0, and glycine/NaOH, pH 8.5–12. All other reaction conditions were as described above. Nuclease activity was apparent throughout the pH range 6–10 with a peak at pH 7.5 (Fig. 5).

Effect of temperature

In a 10 min reaction the optimal temperature was found to be in the range 37–55 °C. Above 55 °C enzyme activity was rapidly lost. Within this temperature range, however, the period of linear phase of the reaction became shorter when temperature was higher. Thus, whereas at 55 °C the linear phase lasted 2.5 min, at 37 °C it lasted at least 15 min (Fig. 6).

Enzyme stability was assessed by measuring activity after a previous treatment at 50 °C. At this temperature the enzyme (6 nM) was stable for 5 min, whereas addition of BSA (100 μg/ml) prolonged activity for a further 10 min. The addition of dimethyl sulfoxide, however, did not significantly affect the stability of the enzyme.

Requirement for thiols

The endonuclease activity was greatly diminished unless dithiothreitol or 2-mercaptoethanol was included in the reaction mixture (Table 2). Concentrations of 1 mM-dithiothreitol or 7 mM-2-mercaptoethanol were used in all of the experiments reported.

Addition of reagents such as 1 mM-Hg²⁺ or 1 mM-iodoacetate completely abolished enzyme activity (Table 2). These results and the loss of activity in the absence of dithiothreitol or 2-mercaptoethanol suggest that the activity requires the enzyme to be in its reduced form, and that certain cysteine residues might be involved in DNA hydrolysis or in the structural stability of the enzyme.

Inhibitor-sensitivity

The addition of chelating agents such as EDTA in the presence of 10 mM-Mg²⁺ partially inhibited the activity at a concentration of 1 mM (10 mM-EGTA produced the same effect; Table 2), further supporting the requirement of bivalent cations for the reaction. Addition of 5'-mononucleotides (dAMP, dCMP, dGMP and dTMP) inhibited activity at concentrations higher than 0.1 mM, whereas the corresponding nucleosides (1 mM) did not affect it (Table 2), thus suggesting that inhibition is caused by the phosphate residue. This supposition was reinforced when 15 mM-phosphate buffer was used instead of 10 mM-Tris/HCl buffer in the reaction (82% inhibition), or when it was used as an inhibitor (62% inhibition with 10 mM-phosphate buffer) in the standard reaction mixture (Table 2). To test the possibility of a chelating effect of phosphate residues on Mg²⁺, we increased the

Fig. 6. Time course of nuclease activity at different temperatures

The activity of the purified enzyme was assayed as described in the Experimental section except that the temperature (○, 25 °C; ●, 30 °C; △, 37 °C; ▲, 45 °C; □, 50 °C; ■, 55 °C) and the time of incubation were varied as indicated in the Figure. Each sample was incubated with 3 nM enzyme and excess of substrate. The enzyme activity obtained under the standard assay conditions (see the Experimental section) was set at 100 %.

Mg²⁺ concentration in the reaction mixture, but found no decrease of inhibition (results not shown).

The effects of other compounds, such as denaturing agents, are also shown in Table 2.

Substrate-specificity

The enzyme could hydrolyse native and denatured DNA, but not tRNA or rRNA. Addition of *E. coli* tRNA to a reaction mixture with DNA inhibited nuclease activity at concentrations higher than 3 μ M (Table 2). The K_m of the enzyme for double-stranded bacteriophage λ DNA was approx. 6 nM, whereas for single-stranded denatured DNA it was approx. 15 nM (2.5-fold greater; for double-stranded and single-stranded bacteriophage M13mp18 DNA the K_m values were approx. 7 nM and 15.3 nM respectively). Thus the affinity of the enzyme for double-stranded DNA was greater than that for single-stranded DNA.

The kinetics of degradation of supercoiled, circular and linear forms of plasmid pBR322 at short times and at low and high enzyme concentrations were analysed. The diluted enzyme at first produced circular forms after the hydrolysis of supercoiled chains, from which a subsequent increase of linear forms of plasmid was observed. This indicated that the enzyme produced nicks in a chain of supercoiled DNA, resulting in open circular forms. When the nicks increased in both chains, linear forms were formed, which were subsequently degraded at higher enzyme concentration (Fig. 7).

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

The property of terminal deoxynucleotidyltransferase to catalyse the addition of deoxyribonucleoside triphosphates to 3'-hydroxy ends of DNA fragments was used to identify the production of 3'-hydroxy termini by *S. glaucescens* nuclease (see the Experimental section). The incorporation of radioactivity (25 %) indicated that the nuclease generated 3'-hydroxy termini

Fig. 7. Cleavage of form I pBR322 DNA to form III via a nicked intermediate

Reaction mixtures were set up, each containing 0.5 μ g of pBR322 DNA (Form I) in 30 μ l of reaction buffer (see the Experimental section). Small amounts (less than 0.01 unit) of the endonuclease were added and the reaction mixtures were incubated at 37 °C for (left to right) 0, 0.5, 1, 2, 2.5, 3, 3.5 and 5 min respectively.

in its cleavage of double-stranded DNA. That the enzyme also generated 5'-phosphate termini was indicated by the following experiment. A digestion of bacteriophage λ DNA by the endonuclease was carried out and the reaction was stopped at an appropriate time. 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 bacteriophage T4 polynucleotide kinase reaction.

DISCUSSION

We have purified a strongly regulated endodeoxyribonuclease that seems to be the only major DNAase activity present in *S. glaucescens*. To our knowledge this is the first report of a purification and characterization of a nutritionally controlled sequence-non-specific DNAase in *Streptomyces*. Enzymes with similar characteristics have been detected and partially characterized in at least five other different species, *Streptomyces lividans* (JI 1326), *Streptomyces violaceoruber* D.S.M. 40783, *Streptomyces coelicolor* A.T.C.C. 10147, *Streptomyces achromogenes* A.T.C.C. 12767 and *Streptomyces albus* G [19] (J. F. Aparicio, S. Cal, J. F. Santos, C. Hardisson & J. Sánchez, unpublished work), and it seems reasonable to conclude that these types of DNAases are common to all *Streptomyces*.

Some of the properties of the enzyme described in the present paper are similar to those of type II restriction endonucleases and other nucleases. Its requirement for Mg²⁺ is similar to that of most of these kinds of enzymes [20,21], and its preference for a low-ionic-strength environment coincides with a number of them [22]. However, its relatively high pI contrasts with those of most type II enzymes, as derived from their behaviour with anion-exchangers. It is noteworthy that the activity is stimulated in the presence of dimethyl sulphoxide, and that this stimulation seems to be carried out through a significant increase in the reaction rate per enzyme, since enzyme stability is not significantly affected.

There are very few studies on these kinds of nucleases in *Streptomyces* [23,24]. Thus, looking for analogies with other

nucleases previously described, we found a remarkable similarity with pancreatic DNAase. *S. glaucescens* DNAase and pancreatic DNAase I share the following common properties: (1) both require Mg^{2+} or other bivalent cations such as Mn^{2+} or Ca^{2+} for activity, and are inhibited at concentrations over 20 mM; (2) both are enzymes specific for DNA [25]; (3) both show preference for double-stranded DNA [26]; (4) both are inhibited at high salt concentration [27]; (5) both generate nicks on double-stranded DNA [28]; (6) both have a similar pH optimum [25] and M_r ; both produce 5'-phosphomonoesters [26]. However, despite the numerous similarities there are a number of properties that distinguish them: the isoelectric point of *S. glaucescens* DNAase (8.15) is clearly different to that of DNAase I (pI 4.7–5.0); their requirements for certain cofactors and thiols and their inhibitor-sensitivities are also different.

The M_r of *S. glaucescens* endonuclease was estimated to be 49 000 by gel filtration. The M_r of the denatured enzyme estimated by SDS/PAGE, however, was 39 600. The significant difference in these two M_r values may be due not only to the fact that this protein does not have to be globular, but to the basic nature of the enzyme protein. It is well known that the apparent M_r values of basic proteins determined by SDS/PAGE are sometimes anomalous [29]. Thus a monomeric nature of the native enzyme can be concluded.

A remarkable feature of the *S. glaucescens* endodeoxyribonuclease, and an important area of further investigation, is the regulation of its activity and the role of the enzyme in the cell, since the regulatory mechanisms that control expression of genes from hydrolytic enzymes in *Streptomyces* are virtually unknown. The availability of purified enzyme should allow for the development of suitable reagents for the further analysis of this system. Development of antibodies should enable us, in conjunction with metabolic labelling, to determine if the increase in enzyme activity is due to the synthesis of new enzyme protein or if the increase is a consequence of modification of a pre-existing catalytically inactive protein. Antibodies should also prove useful in screening libraries in an effort to clone the gene encoding the enzyme. The purified enzyme should also enable us to study its mode of interaction with DNA. As this is the first purification and characterization of a DNAase with a nutritional regulation in *Streptomyces*, it may serve as a model system for elucidation of the exact mechanism and role of this regulation in these bacteria.

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