Properties of the major non-specific endonuclease from the strict anaerobe *Fibrobacter succinogenes* and evidence for disulfide bond formation *in vivo*

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DNase A is a non-specific endonuclease of *Fibrobacter succinogenes*. The enzyme was purified to homogeneity and its properties studied both *in vitro* and *in vivo*. Magnesium but not calcium was essential for nucleolytic activity. Manganese ions substituted for magnesium but were less stimulatory. DNase A activity was markedly inhibited by either NaCl or KCl at concentrations greater than 75 mM. The enzyme had a temperature optimum of 25 °C and a pH optimum of about 7.0. Values for K_m and K_{cat} were determined to be 61 µM and 330 s⁻¹ respectively, with a catalytic efficiency approximately threefold greater than bovine pancreatic DNase I, but 10-fold less than the *Serratia marcescens* NucA. DNase A was localized to the periplasm and probably exists as a monomeric species. The enzyme possessed one or more disulfide bonds. In the reduced form it had an apparent mass of 33 kDa, while in the oxidized form it was 29 kDa as estimated by SDS-PAGE. Reduction of the disulfide bonds by dithiothreitol with or without subsequent alkylation by iodoacetamide strongly inactivated the enzyme. DNase A accumulated *in vivo* had an apparent mass of 29 kDa, indicating that it was in an oxidized form. This is the first indication in a strict anaerobe of a functional periplasmic disulfide bond forming system, phenotypically similar to Dsb systems in facultative and aerobic bacteria.

Keywords: endonuclease, anaerobe, disulfide bond, *Fibrobacter succinogenes*

INTRODUCTION

Fibrobacter succinogenes has been cited as an important cellulose degrading micro-organism in the bovine rumen (Chesson & Forsberg, 1997; Halliwell & Bryant, 1963) and one of the most cellulolytic organisms isolated from any environment (Weimer, 1996). Although numerous cellulolytic and hemicellulolytic enzymes have been characterized, continued research into deciphering the mechanisms by which *F. succinogenes* degrades plant cell wall polymers has been hampered by the lack of a gene transfer system. Cited as a possible barrier to transformation by DNA, the DNase A protein was previously detected in partially purified cell lysates and characterized as a non-specific endonuclease (Lee *et al.*, 1992). The enzyme can cleave double and single stranded

DNA, and *F. succinogenes* genomic DNA, to fragments primarily 8–11 nt in length. In a continued effort to characterize this enzyme and investigate its possible negative influence on transformation by DNA, we have purified DNase A to homogeneity from *F. succinogenes* cells and report some catalytic properties of the enzyme.

In addition, we have had the opportunity to extend our investigation into aspects of disulfide bond formation in *F. succinogenes* because DNase A migrates more slowly during SDS-PAGE when the disulfide bonds are reduced, and the reduced and oxidized states of the enzyme can be readily resolved by gel electrophoresis. We are unaware of any disulfide bond formation studies using strict anaerobes and unlike the organisms traditionally used in such studies, *F. succinogenes* is a strictly anaerobic bacterium that requires a well-poised low-redox environment for growth. The question therefore was whether an extracytoplasmic enzyme of this bacterium would be efficiently oxidized *in vivo*.

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METHODS

Bacterial strain and growth. *F. succinogenes* strain S85 was cultured in a liquid medium (Scott & Dehority, 1965) with glucose as the carbon source at a concentration of 0.5% (w/v), and with cysteine-HCl at 5.7 mM as the reducing agent. Inoculation of cultures was conducted under a stream of oxygen-free CO₂ and vessels were tightly stoppered to maintain anaerobicity. Unstoppered vessels and Petri plates containing medium solidified with 1.5% (w/v) agar (Difco Laboratories) were manipulated in an anaerobic chamber (Coy) under a 90% (v/v) CO₂ and 10% (v/v) H₂ atmosphere. The growth temperature was 37 °C.

Cellular fractionation and subcellular distribution of enzyme activities. Cells were fractionated by a process based on that of Gong & Forsberg (1993). Cells were collected by centrifugation (3000 g, 15 min, 4 °C) from a 120 ml volume of culture after growth for 18 h to a density of 0.8 mg cells (dry wt) ml⁻¹. The cell-free extracellular culture fluid was retained. The cell pellet was mixed to form a thick slurry and 50 ml ice-cold distilled water was added. The cells were vigorously resuspended in the water and stored on ice for 15 min, after which time they were again collected by centrifugation. The cell-free supernatant was retained and the cell pellet was again mixed to form a thick slurry, and then vigorously suspended in 50 ml 50 mM Tris/HCl (pH 7.5) buffer containing 0.5 M NaCl. After incubation on ice for 15 min, the cells were collected by centrifugation, the supernatant was retained, and the Tris/HCl/NaCl wash was repeated two additional times. The supernatants from the distilled water wash and the three salt washes that contained periplasmic contents and outer membrane were pooled (fraction 1). The treated cells were suspended in 20 ml 50 mM Tris/HCl (pH 7.5) and subjected to three passes through a French Press (16000 p.s.i. [110400 kPa], fraction 2). A 20 ml volume of cell culture (including the culture supernatant) abstracted from the original overnight culture was subjected to three passes through a French Press and this fraction was labelled whole cell lysate. All fractions were concentrated ninefold relative to each originating culture volume through a PM-10 membrane (Amicon, 10 kDa molecular mass cut-off). The concentrated samples were centrifuged (16000 g, 20 min, 4 °C), decanted and stored on ice at 4 °C. From fractions 1 and 2, 1 ml aliquots were retained and the remaining volumes were ultracentrifuged (100000 g, 4 °C) for 2.5 h to sediment membranous materials. The cytoplasmic membranes from fraction 2 were resuspended in 1 ml 20 mM PIPES (pH 6.8) buffer and diluted when assayed for enzyme activities. A smaller pellet was collected from fraction 1, and both this pellet (labelled outer membrane) and the supernatant (labelled periplasm) were handled as described for the cytoplasm and cytoplasmic membrane samples. Equivalent volumes of the fractions and the whole cell lysate were assayed for nuclease activity (microtitre plate assay), glutamate dehydrogenase (cytoplasmic marker), succinate dehydrogenase (cytoplasmic membrane marker) and cellobiosidase (periplasmic marker), and for total protein using the method of Bradford (1976).

Nuclease assays. Three different methods were used to evaluate nuclease activity. Nuclease activity was compared between crude samples by monitoring the decrease in fluorescence of a herring testes DNA/ethidium bromide solution when the DNA was depolymerized in a microtitre plate assay (Ball *et al.*, 1990). After incubation, the microtitre plate was viewed over a UV transilluminator and the titre of nuclease activity was scored visually by recording the last well in a series that demonstrated a complete loss of ethidium bromide fluorescence due to DNA degradation. In some cases, in-

complete loss of fluorescence in the last well in a series would be scored as one half loss of fluorescence. Activity was expressed as the inverse of the titre. Nuclease activity in the presence of various salts could also be qualitatively demonstrated by using agarose gel electrophoresis and ethidium bromide staining to detect the loss of a DNA substrate after exposure to nuclease. Gels contained 0.8% agarose and the running buffer was TBE (45 mM Tris-borate, 2 mM EDTA, pH 8.0). The hyperchromicity (Kunitz) assay was used for pure or partially purified protein samples and detects the increase in absorbance by DNA at 260 nm when the polymer is depolymerized to nucleotides or short oligonucleotides (Friedhoff *et al.*, 1996; Kunitz, 1950). Assays were conducted with a reagent mixture containing 50 mM Tris/HCl (pH 7.5), 20 mM MgCl₂ and 40 µg herring testes DNA ml⁻¹ (Sigma), unless otherwise noted. The reaction volume was 450 µl and 0.25 µg purified DNase A was used per assay unless otherwise noted. Assays were conducted in triplicate and regression curves were generated from the linear portion of each progress curve. Specific activity was reported as $\Delta A_{260} \text{ min}^{-1} (\text{mg protein})^{-1}$. Assays were conducted at 22 °C unless otherwise noted. The influence of pH on nuclease activity was tested using a three-buffer mixture [0.1 M ACES ($pK_a = 6.65$), 0.052 M Tris ($pK_a = 8.00$), 0.052 M ethanolamine ($pK_a = 9.47$)] at 30 °C (Ellis & Morrison, 1982). Each experiment was conducted at least twice.

Other enzyme assays. With the exception of the nuclease assays previously described, one unit is defined as the conversion of 1 nmol substrate min⁻¹. Specific activity was defined as units (mg protein)⁻¹. Glutamate dehydrogenase activity was measured using a protocol based on that of Malamy & Horecker (1961). The reagent mixture contained 20 mM Tris/HCl (pH 7.7), 10 mM MgCl₂, 0.8 mM NADP⁺ and 5 mM sodium glutamate (final concentrations). Initial rates of reaction were calculated by using an extinction coefficient of 6200 M⁻¹ cm⁻¹ for NADPH (Dawson *et al.*, 1986). Cellobiosidase activity was measured spectrophotometrically using the cellobiose analogue *p*-nitrophenyl cellobioside as substrate, as described by Huang & Forsberg (1988). Succinate dehydrogenase activity was measured as described by Dickie & Weiner (1979) by monitoring the phenazine methosulfate coupled reduction of 3(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT). Initial rates of reaction were calculated by using an extinction coefficient of 17000 M⁻¹ cm⁻¹ for reduced MTT (Weiner, 1974).

Nuclease purification. After growth at 37 °C for 18 h, cells from 12 l medium were collected under aerobic conditions by centrifugation at 13200 g for 20 min at 4 °C. The resultant pellets were pooled and the periplasmic contents were released as described above. The concentrated periplasmic fraction was ultracentrifuged at 100000 g for 120 min at 4 °C. The supernatant was recovered and brought to 80% saturation with solid ammonium sulfate; the pH was maintained near neutrality with the addition of 1 M NH₄OH as needed. The preparation was stirred on ice for 45 min, after which proteins were recovered by centrifugation at 13200 g for 60 min at 4 °C. The supernatant was discarded and protein pellets were resuspended in TE buffer (20 mM Tris/HCl, pH 7.3 at 4 °C, 0.5 mM EDTA) and mixed until the proteinaceous material was in suspension. This protein solution was concentrated and desalted (via buffer exchange) through a PM-10 Amicon membrane. The concentrated, desalted protein solution was applied to a chromatography column (20 × 1.5 cm) containing Heparin Sepharose CL-6B (Amersham Pharmacia Biotech) that had previously been equilibrated with TE buffer. Protein was eluted from the medium with a 300 ml 0.2–0.9 M NaCl

gradient and a flow rate of 0.4 ml min^{-1} . Fractions were tested for conductivity (NaCl concentration), A_{280} (protein concentration), and nuclease activity as detected by an SDS-PAGE–DNA zymogram (Lee *et al.*, 1992). Fractions containing nuclease activity were pooled, concentrated and desalted via buffer exchange through a PM-10 Amicon membrane. The concentrated and desalted protein sample was applied to a $20 \times 1.5 \text{ cm}$ chromatography column containing hydroxyapatite Bio-Gel HTP (Bio-Rad) equilibrated with 20 mM potassium phosphate buffer (pH 7.3 at 4°C). Proteins were eluted using a 300 ml $0.1\text{--}0.6 \text{ M}$ potassium phosphate buffer (pH 7.3) gradient and a flow rate of 0.4 ml min^{-1} . Fractions containing nuclease activity were pooled and desalted using a PM-10 Amicon membrane and the phosphate buffer was exchanged with the TE buffer.

The protein sample was applied to a $90 \times 1 \text{ cm}$ column containing Sephadex G-75 (Amersham Pharmacia Biotech) that had previously been equilibrated in the TE buffer. Proteins and buffer were carried through the column under the force of gravity with a flow rate of 0.04 ml min^{-1} . Appropriate fractions were pooled and concentrated and this final preparation was assessed for purity by SDS-PAGE (Laemmli, 1970) followed by staining with Coomassie brilliant blue R-250 and by silver staining. Protein samples were stored on ice at 4°C or were supplemented with 25% (w/v) glycerol and stored at -20°C .

Determination of molecular mass by size-exclusion chromatography. DNase A was applied to a $90 \times 1 \text{ cm}$ chromatography column containing Sephadex G75 (Amersham Pharmacia Biotech) previously equilibrated with TE buffer. The column was calibrated using a Low Molecular Mass Gel Filtration Calibration Kit (Amersham Pharmacia Biotech) according to the manufacturer's directions. Proteins were carried through the column under the force of gravity with a flow rate of $0.04 \text{ ml TE min}^{-1}$.

DNase A kinetic characteristics. To calculate values for K_m and $V_{\max}$, initial velocities were measured via the Kunitz assay at various DNA concentrations. Data were fitted to the Michaelis–Menten equation and values for K_m and $V_{\max}$ were estimated using a SigmaPlot 2.01 (SPSS Science) curve-fitting program that fits data to non-linear functions directly by non-linear regression (Kuo, 1992).

To compare the parameters of K_m and K_{cat} for DNase A to various other nucleases, K_m values were converted from $\mu\text{g ml}^{-1}$ to μM internucleotide bonds (Hale *et al.*, 1993) by using a mean molar mass of $330 \text{ g mol}^{-1} \text{ nt}^{-1}$. $V_{\max}$ values were converted to K_{cat} by assuming the molar mass per nucleotide was 330 g mol^{-1} and that a change in absorbance of 0.3 accompanied the degradation of 0.05 mg DNA. The molecular mass of DNase A was assumed to be 33 kDa. K_{cat} was obtained from the general expression $V_{\max} = K_{\text{cat}}[E_0]$.

Influence of disulfide bond formation on DNase A activity. Equivalent amounts of enzyme in 50 mM Tris/HCl (pH 8.0) were reduced with 25 mM DTT for 30 min at 37°C with or without a subsequent supplementation of 30 mM iodoacetamide and further incubation for 15 min at room temperature. Identical samples were either untreated (control) or treated with 30 mM iodoacetamide alone and incubated for 15 min at room temperature. After incubation, excess reagents in all samples were removed by centrifugation through a 10 kDa molecular mass cut-off membrane (PM-10, Amicon). Protein concentrations were determined and samples were adjusted to the appropriate volume in buffer and assayed for activity via the Kunitz assay. Aliquots of each sample were

also resolved by SDS-PAGE and the electrophoretic mobilities of the treated proteins were compared with those of reduced and oxidized standards of DNase A.

Immunological techniques. Polyclonal antiserum was raised against purified antigen by injection into a New Zealand White rabbit. Antibodies specific for DNase A were purified from serum by a method modified from that of Olmstead (1986). Antigen was detected by either the alkaline phosphatase detection system (Bio-Rad) as described by the manufacturer or by a horseradish peroxidase based Super-Signal Ultra (Pierce) chemiluminescence system as described by the manufacturer.

Gel electrophoresis. SDS-PAGE was conducted using 12% polyacrylamide gels (Laemmli, 1970) at 4°C and 120 V for 1 h. When SDS-PAGE was conducted under oxidizing conditions, reducing agents were omitted from the electrophoresis sample buffer and samples were not heated. For molecular mass determination via SDS-PAGE, Broad-Range molecular mass protein standards (Bio-Rad) were used. Standard reduced DNase A was produced by treatment of the enzyme with 10 mM DTT for 30 min followed by addition of iodoacetamide to a final concentration of 25 mM and a further incubation of 15 min at 37°C . Excess reagents were removed by filtration. DNase A purified under ambient atmospheric conditions was obtained as an oxidized species and this was used as the standard oxidized protein. Immediately before electrophoresis, appropriate volumes of each standard were mixed together and further mixed with an equal volume of sample buffer lacking reducing agent. When applied to a single lane and electrophoresed, two bands with apparent molecular masses of 33 kDa and approximately 29 kDa were detected, representing the reduced and oxidized forms of DNase A, respectively.

SDS-PAGE–DNA zymograms were conducted as above except that $100 \mu\text{g}$ salmon sperm DNA ml^{-1} (final concentration) (Difco Laboratories) was added to the gel preparation before polymerization and reducing agents were omitted from the sample-loading buffer. The samples were not heated prior to loading on the gel. After electrophoresis at 120 V, gels were washed and incubated as described by Lee *et al.* (1992). The development of activity was detected by staining the gel in an aqueous solution of ethidium bromide ($0.5 \mu\text{g ml}^{-1}$) for 20 min, destaining the gel for 20 min in incubation buffer and viewing the gel over a UV transilluminator. Non-fluorescent bands against a fluorescent background indicated enzymic activity. Images of the electrophoretic gels were captured using a Gel-Doc 1000 gel documentation system (Bio-Rad) and were subsequently scanned on a ScanJet 6100C scanner (Hewlett Packard).

Protein determination. Protein concentration was determined either by the method described by Bradford (1976) or the bicinchoninic acid protein assay (Smith *et al.*, 1985) using commercially available reagents from either Bio-Rad or Sigma, respectively. Bovine serum albumin was used as the reference protein.

Determination of *in vivo* redox state of DNase A. A 150 ml culture of *F. succinogenes* was grown for 24 h. At time points roughly corresponding to lag phase, exponential growth phase, late exponential phase, early stationary phase and decline phase, aliquots of cell suspension were withdrawn from the bulk culture and total cell protein concentrations were determined using the bicinchoninic acid method. Within each sample the redox state of DNase A was assessed essentially as described by Kishigami *et al.* (1995), except that

all manipulations prior to electrophoresis were conducted in an anaerobic chamber. DNase A was detected by chemiluminescent Western blotting. The relative mobility of DNase A in each sample was compared with reduced and oxidized standards of DNase A. Redox measurements of growth media were made with an Orion SA250 pH meter equipped with a Corning Redox Combo w/RJ redox probe (Orion Research).

RESULTS AND DISCUSSION

Cell fractionation and nuclease localization

After subcellular fractionation, most of the glutamate dehydrogenase activity (73 % of the activity recovered from the subcellular fractions) and all of the succinate dehydrogenase activity was detected in the cytoplasm and cytoplasmic membrane fraction, respectively (Table 1). Cellodextrinase, which was known to be a periplasmic enzyme (Gong & Forsberg, 1993), partitioned with the periplasmic fraction. These data indicate that the cell fractionation procedure was relatively efficient.

Testing of each fraction for nuclease activity showed that almost all was present in the periplasmic fraction (Table 1). When fractions were probed with DNase A-specific antiserum, the major signal was detected in the periplasmic fraction and a much weaker signal was detected in the cytoplasm (not shown). These data demonstrate that DNase A is a periplasmic protein. This location may allow the nuclease to adequately carry out its presumed function as a barrier to the uptake of foreign DNA by intercepting and degrading the polymer before passage into the cytoplasm occurs.

Nuclease purification and molecular mass determination

Table 2 shows the purification process as monitored by nuclease assays and total protein. The enzyme was purified 550-fold. From 12 l cell culture utilized in this experiment, 100 µg pure protein was obtained. Over the course of this work 130 l overnight culture were

Table 1. Distribution of nuclease activity, glutamate dehydrogenase, succinate dehydrogenase, cellodextrinase and protein in subcellular fractions

ND, Not detected. Values in parentheses are percentage activity expressed relative to that of the whole cell lysate that is taken as 100 %. Standard errors less than 0.1 are not reported.

Fraction	Distribution of enzyme activity or protein concentration (%)				
	Nuclease*	Glutamate dehydrogenase†	Succinate dehydrogenase†	Cellodextrinase†	Protein (mg ml ⁻¹)
ECCF‡	34 (< 1)	ND (0)	ND (0)	ND (0)	0.2 (4)
Outer membrane	60 (1)	ND (0)	ND (0)	ND (0)	0.1 (3)
Periplasm	4860 (100)	58 ± 3 (24)	ND (0)	362 ± 14 (71)	1.5 (29)
Cytoplasmic membrane	61 (1)	8 ± 1 (4)	244 ± 2 (108)	17 ± 3 (3)	1.1 (22)
Cytoplasm	541 (11)	182 ± 20 (60)	ND (0)	124 ± 3 (21)	1.7 (34)
Whole cell lysate	4860 (100)	300 ± 30 (100)	225 ± 10 (100)	580 ± 10 (100)	5.0 (100)

* Nuclease specific activity is the inverse of the titre obtained in the microtitre plate assay.

† Enzyme activity is the mean ± standard error of the total activity.

‡ Extracellular culture fluid.

Table 2. Purification of the nuclease

Total activity is expressed as ΔA_{260} units min⁻¹ and specific activity was ΔA_{260} units min⁻¹ (mg protein)⁻¹.

Step	Total activity*	Total protein (mg)	Specific activity [U(mg protein) ⁻¹]
Periplasmic precipitate	603	861	0.7
Heparin sepharose	292.5	37	7.9
Hydroxyapatite	198	8	24.8
Gel filtration	49.5	0.1	495

* Assays were conducted with 25 µg DNA ml⁻¹ at 25 °C.

Fig. 1. SDS-PAGE and Coomassie brilliant blue staining of protein samples after each step in the purification process. Lane M, broad range molecular mass markers (kDa); lane 1, periplasmic proteins; lane 2, after heparin Sepharose chromatography; lane 3, after hydroxyapatite gel chromatography; lane 4, after Sephadex G75 size exclusion chromatography. Approximately 3 μ g purified DNase A was loaded into lane 4. Note: all protein samples were mixed with the Laemmli sample buffer containing SDS and β -mercaptoethanol and heated prior to electrophoresis, a process that reduces disulfide bonds.

processed for a final yield of 1 mg pure protein. Fig. 1 demonstrates the purification process as monitored by SDS-PAGE after treatment of protein preparations with sample buffer containing β -mercaptoethanol and staining with Coomassie brilliant blue R-250. Silver staining of the pure preparation detected a single band of 33 kDa, demonstrating purification to homogeneity. The molecular mass of DNase A by size exclusion chromatography on Sephadex G-75 was estimated to be 26 kDa, which is similar to the 29 kDa monomeric mass of DNase A inferred from SDS-PAGE, suggesting that the enzyme exists as a monomer in solution.

Characterization of DNase A

The optimum concentration of Mg^{2+} ions for maximum activity in the Kunitz assay was broad and extended from approximately 10 to 30 mM $MgCl_2$; reagent mixtures were usually supplemented with 20 mM $MgCl_2$. No activity was exhibited in the absence of added cation. The rate of DNA depolymerization was insensitive to whether the conjugate anion to Mg^{2+} was Cl^- or SO_4^{2-} and 20 mM Mn^{2+} resulted in a 2.5-fold greater specific activity [$1550 \pm 250 A_{260}$ units min^{-1} (mg protein) $^{-1}$] than that exhibited with 20 mM Mg^{2+} [$600 \pm 48 A_{260}$ units min^{-1} (mg protein) $^{-1}$]. Ca^{2+} did not

Fig. 2. Nuclease activity in the absence of cation (no cation) and in the presence of various cations of chloride salts was detected by agarose gel electrophoresis of the reaction products. Equivalent amounts of herring sperm DNA was supplemented with 5 mM salt (shown above each pair of lanes) and incubated in the absence (lanes 1) or presence (lanes 2) of 0.25 μ g DNase A for 3 min prior to stopping the reaction by addition of 50 mM EDTA and electrophoresis. Remaining substrate was visualized by ethidium bromide staining after electrophoresis. Undigested DNA is visible as a high-molecular-mass smear in the gel, except in the presence of zinc or copper salts (see text).

appreciably stimulate activity. When other transition divalent cations were used to substitute as required cofactors in the catalytic mechanism of DNase A using the Kunitz assay, uninterpretable results were obtained. For example, supplementation of the assay mixture with 5 mM $CoCl_2$ led to a rapid, linear, enzyme-independent increase in A_{260} – a condition that, without proper controls, could be mistaken for enzyme activity. We therefore confirmed the spectrum of stimulatory cations in the Kunitz assay by using an agarose-gel-based assay and visually assessing the degradation of DNA substrate after exposure to nuclease in the presence of various cations. In the absence of cation (Fig. 2) there was little nuclease-dependent substrate depletion as indicated by the perseverance of the high molecular mass DNA smear. Supplementation with Mg^{2+} and nuclease (lane 2 of the magnesium treatment) resulted in complete depolymerization of the DNA in contrast to that observed in the control treatment where only the Mg^{2+} was added (lane 1 of the magnesium treatment). For Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} , the appearance of the DNA smear is similar for both the control treatment (salt only; lanes 1) and the test treatment (salt and nuclease; lanes 2). Therefore, supplementation with these salts does not lead to enzyme-dependent degradation of the DNA. When nuclease is added to the DNA treated with calcium cations (lane 2), there appears to be a small decrease in polymer size but this effect is similar to when the DNA is exposed to nuclease in the absence of any cation. When the nuclease was tested for activity via the Kunitz assay in the absence of added cation or in the presence of calcium, there was a very small, almost negligible increase in absorbance, perhaps indicating very low levels of basal activity under these conditions. Interestingly, the gel-based assay demonstrated that Mn^{2+} stimulated activity less than did Mg^{2+} , in contrast

Fig. 3. Effect of substrate concentration on DNase A activity. Nuclease activity on herring testes DNA was monitored by the Kunitz assay. Line was fitted to data and values for K_m ($20 \pm 4 \mu\text{g DNA ml}^{-1}$) and $V_{\max}$ [$1192 \pm 88 A_{260} \text{ units min}^{-1} (\text{mg protein})^{-1}$] were calculated by SigmaPlot curve fitting program using non-linear regression. Mean values and standard errors were calculated from three replications.

to the data generated using the Kunitz assay. The failure to corroborate the Kunitz assay data by visual detection of DNA degradation as monitored by ethidium bromide staining (for Mn^{2+}) may be a consequence of the rather well-documented effects that certain (especially transition) cations have on DNA structure (for examples, see Anderson *et al.*, 1971; Kejnovsky & Kypr, 1998; Langlais *et al.*, 1990) and therefore its UV absorption characteristics in the Kunitz assay. In Fig. 2, DNA supplemented with Cu^{2+} and Zn^{2+} [either without (lane 1) or with (lane 2) added nuclease] also appeared differently after electrophoresis and staining, perhaps suggesting a structural change in the DNA. Obviously, caution is needed when assessing the ability of a metal to act as a co-factor

with any nuclease assay. Our data indicates that DNA depolymerization does not appreciably take place in the absence of magnesium or manganese, or in the presence of calcium, nickel, zinc or copper salts. With regard to the latter salts, we did not differentiate whether these ions are simply not permissible co-factors, are inhibitory to the nuclease or their effect on DNA renders the substrate resistant to cleavage by nuclease.

The optimal temperature for activity was demonstrated to be approximately 25°C [$810 \pm 10 A_{260} \text{ units min}^{-1} (\text{mg protein})^{-1}$] with greater than 75 % of the maximum activity at both 20 and 33°C . At a temperature corresponding to the *in situ* rumen temperature of about 39°C , the enzyme retained only about 33 % of the activity exhibited at 25°C . This may reflect differences between the *in vivo* and *in vitro* behaviour of the enzyme but this enzyme is one of several *F. succinogenes* hydrolases, for example, endoglucanase G (Iyo & Forsberg, 1999), that have curiously low optimum temperatures for activity *in vitro*.

Enzymic activity was at the maximum at a pH of 7.0, but retained greater than 90 % of that activity between pH values of 6.5 and 8.7.

NaCl and KCl were used to assess the effects of monovalent cations on nuclease activity. Responses to both chemicals were similar and neither salt significantly stimulated activity. Activity remained constant between 0 and 75 mM but 100 mM was slightly inhibitory and at a concentration of 300 mM the enzyme exhibited less than 20 % of its activity in the absence of these salts.

The rate of DNA depolymerization catalysed by DNase A as a function of substrate concentration followed Michaelis–Menten kinetics (Fig. 3). A K_m value of $20 \pm 4 \mu\text{g DNA ml}^{-1}$ and a $V_{\max}$ value of $1192 \pm 88 A_{260} \text{ units min}^{-1} (\text{mg protein})^{-1}$ were estimated from the curve and these values were converted to a K_m of $61 \pm 12 \mu\text{M}$ and a K_{cat} of $330 \pm 24.5 \text{ s}^{-1}$, respectively. These values are compared with previously published kinetic quantities for other nucleases in Table 3. DNase

Table 3. Comparison of the catalytic properties of DNase A with other endonucleases

Values for kinetic parameters of other enzymes are from Friedhoff *et al.* (1996) and Meiss *et al.* (1998).

Enzyme	Substrate DNA	K_m (μM)*	K_{cat} (s^{-1})	K_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	Preferred co-factor	Reference
<i>F. succinogenes</i> DNase A	Herring testes	61 ± 12	330 ± 25	5.4	Mg^{2+}	This work
<i>S. marcescens</i> NucA	Salmon testes	53.8 ± 8	2100 ± 63	54	Mg^{2+}	Friedhoff <i>et al.</i> (1996)
Bovine pancreas DNase I	Calf thymus	109	176	1.6	Mg^{2+}	Doherity <i>et al.</i> (1995)
<i>Staphylococcus</i> nuclease	Salmon sperm	48 ± 11	730 ± 76	15	Ca^{2+}	Poole <i>et al.</i> (1991)
<i>Anabaena</i> NucA	Herring sperm	164	1921	11.7	Mn^{2+} , Co^{2+}	Meiss <i>et al.</i> (1998)

* Determined using the Kunitz assay.

Fig. 4. Differential migration of oxidized and reduced DNase A in an SDS-PAGE-DNA zymogram. Before electrophoresis, equivalent aliquots of French Press-disrupted whole cells were incubated for 30 min at 37 °C in sample loading buffer lacking a reducing agent (lane 1) or in sample buffer supplemented with either 1.4 M β -mercaptoethanol (lane 2) or 0.2 M dithiothreitol (lane 3). Positions of molecular mass markers (kDa) are indicated. Only one band of enzyme activity was observed in each of the three treatments.

A had a lower catalytic efficiency than the *S. marcescens* NucA, but a more similar efficiency to the *Staphylococcus* and *Anabaena* nucleases.

Detection and importance of disulfide bonds in DNase A

Beyond efficient secretion out of the cytoplasm, *F. succinogenes* cells must possess a means of keeping the nuclease inactive while in the cytoplasmic compartment since DNase A degrades host genomic DNA. The extracellular nuclease of *S. marcescens* has been shown to require disulfide bonds for catalytic activity (Ball *et al.*, 1992). Since the cytoplasm of *S. marcescens* has a low redox potential, the enzyme is inactive and consequently the cells survive in the presence of intracellular intact, but inactive enzyme (Ball *et al.*, 1992; Benedik & Strych, 1998). It seemed possible that the inability to form disulfide bonds in the reducing cytoplasm could also explain why the nuclease is not lethal in *F. succinogenes*. Experiments were therefore designed to determine whether DNase A possessed disulfide bonds and whether these bonds were required for nucleolytic activity. Whole cell lysates were incubated in SDS-PAGE sample buffer supplemented with either DTT or β -mercaptoethanol, or in sample buffer lacking reducing agents and were subsequently resolved by SDS-PAGE and nuclease activity detected by DNA zymogram analysis (Fig. 4). Non-fluorescent bands indicating nuclease activity developed slowly after treatment with reducing agent and migrated with an apparent molecular mass of 33 kDa. Non-reduced lysate developed a non-fluorescent band much more quickly and this band migrated with an apparent molecular mass of 29–30 kDa. This mobility shift is indicative of one or more disulfide bonds in DNase A.

Purified DNase A was subsequently tested for activity in the Kunitz assay before [$620 A_{260}$ units min^{-1} (mg protein) $^{-1}$] and after treatment with 25 mM DTT to determine whether disulfide bonds were required for

activity. The treatment decreased the activity of the enzyme by 86 %. Reduced proteins can be alkylated with iodoacetamide to modify free thiol groups such that disulfides cannot be re-acquired in a protein after removal of the reducing agent (Kishigami *et al.*, 1995). Alkylation alone of DNase A caused only an 8 % decrease in activity. When the enzyme was alkylated after reduction, the activity of the enzyme was decreased by 96.6 % as compared to that of the unreduced, unalkylated enzyme. Aliquots of treated and untreated samples were resolved by SDS-PAGE to compare the migration of DNase A with oxidized and reduced controls. The migration end points of each sample matched expectations given the treatment applied (i.e. oxidized protein was 29 kDa and the reduced protein was 33 kDa) (not shown). Therefore, DNase A is nearly inactive in the reduced state.

Redox state of DNase A *in vivo*

The initial observation that DNase A was inactivated after reduction by either β -mercaptoethanol or dithiothreitol led to the question of whether the enzyme in growing cells might be present in a reduced and therefore inactive state. This seemed plausible since the cysteine residues within DNase could be reduced because of the combination of a reducing agent in the medium and the well poised low redox potential of the medium during growth. Kobayashi & Ito (1999) indicated that inclusion of dithiothreitol in the culture medium of *Escherichia coli* causes reduction of the periplasmic thiol:disulfide oxidoreductase (DsbA). Similarly, the cysteine included in the medium as a reducing agent might diffuse into the periplasm of *F. succinogenes* and reduce disulfides.

Redox measurements of uninoculated anaerobic media and of *F. succinogenes* cultures showed that the E_h varied from approximately -207 mV for freshly prepared uninoculated medium to approximately -327 mV after 24 h growth. We therefore sought to determine whether DNase A accumulated in a reduced or oxidized form *in vivo*, especially under the stringent reducing conditions ambient after several hours of cell growth in the anaerobic medium. Aliquots of cells were removed from a growing culture at the time points indicated in Fig. 5(a). The whole-cell proteins were immediately precipitated with TCA and modified by iodoacetamide in order to trap reduced proteins. Equivalent amounts of total cell protein from each time point were resolved by SDS-PAGE. DNase A was detected by chemiluminescent Western immunoblotting and it was observed to migrate to a position of 29 kDa, similar to that of an oxidized DNase A control (Fig. 5b). Therefore in spite of the reducing potential of the medium, *F. succinogenes* cells possessed the ability to oxidize periplasmic DNase A molecules throughout 24 h growth in batch culture (Fig. 5) even as the culture medium becomes increasingly and strongly reducing. This suggests that an oxidizing pathway for disulfide bond formation is not compromised by the extremely low redox potential exhibited in the culture medium during

Fig. 5. Redox state of DNase A accumulated *in vivo* during growth. (a) Total cellular proteins extracted from a growing culture (time points 1–5) were precipitated with trichloroacetic acid and treated with iodoacetamide to trap free thiol groups. (b) Precipitated and alkylated proteins from cells removed at each time point in Fig. 5a (1–5) were resuspended and resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with affinity-purified anti-DNase A antiserum and goat anti-rabbit horseradish peroxidase conjugated antibodies. Mobility of DNase A sampled from each stage of growth was detected by chemiluminescence and compared with the mobility of reduced and oxidized standards of DNase A (M).

growth. More importantly, this establishes that *F. succinogenes* possesses a periplasmic mechanism of oxidatively forming and maintaining disulfide bonds in secreted proteins since all proteins initially exist in the cytoplasm in a reduced form. These results provide the first instance of demonstrating *in vivo* periplasmic disulfide bond formation in a strictly anaerobic bacterium though we suspect that the activity is as widespread in this class of bacteria as it seems to be in the facultative and aerobic bacteria.

During growth under aerobic conditions, molecular oxygen is presumed to provide the ultimate oxidizing potential that drives disulfide bond formation, and in the absence of oxygen other common electron acceptors presumably fulfil this role. Clearly, under the anaerobic and reducing conditions typical for the growth of strict anaerobes, a different pathway for the disposal of electrons must be imagined. Bader *et al.* (1999) have recently shown that when a strain of *E. coli* with null

mutations in cytochrome *bd* and *bo* oxidases was grown under anaerobic conditions in a GasPak Anaerobic System (albeit with no added reducing agent) to avoid interference from trace quantities of oxygen and with glucose as the primary carbon source, the periplasmic indicator protein β -lactamase was maintained in an oxidized form. This and related data documented that in *E. coli* growing under anaerobic conditions, the DsbA–DsbB system shuttles electrons via menaquinone to terminal acceptors such as fumarate.

F. succinogenes also possesses a membrane-associated NADH-dependent fumarate reductase that transfers electrons from a membrane receptor, presumably menaquinone, to fumarate with the rather prolific formation of the reduced product succinate (Meinhardt & Glass, 1994). Therefore, this strict anaerobe probably possesses a disulfide bond forming mechanism analogous to that reported for *E. coli* and other facultatively anaerobic and aerobic bacteria. The observation that the DNase A protein remains oxidized despite a redox potential of less than -300 mV demonstrates that the disulfide bond forming system is functional under conditions that are quite reducing, at least in the external medium. Undoubtedly, further detailed experiments will be required to determine whether disulfide bond formation in strict anaerobes involves novel molecular adaptations to the strongly reducing environmental conditions that many other species cannot tolerate.

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