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Isolation, Characterization, and Activation of the Magnesium-Dependent Endodeoxyribonuclease from *Bacillus subtilis*†

William F. Burke, Jr.,* and John Spizizen

ABSTRACT: A major endodeoxyribonuclease was isolated from a mutant of the transformable *Bacillus subtilis* 168. The magnesium-dependent endonuclease was purified approximately 750-fold to electrophoretic homogeneity. The enzyme had a molecular weight of about 31 000, as determined by gel filtration and polyacrylamide gel electrophoresis. The protein appears to be composed of two subunits. The nuclease was dependent on magnesium or manganese ions for hydrolytic activity. The purified nuclease degraded DNA from several species of *Bacillus,* as well as *Escherichia coli* DNA, alkylated, depurinated, and thymine-dimer containing *B. subtilis* DNA, and hydroxymethyluracil-containing phage DNA. The enzyme also hydrolyzed single-stranded DNA, although native DNA was the preferred substrate. However, the nuclease was unable to degrade ribosomal RNA. The cleavage products of the DNA hydrolysis have 5′-phosphate and 3′-hydroxyl ends. The enzyme could be activated in crude extracts by heat treatment or treatment with guanidine hydrochloride. The nuclease activity was inhibited by phosphate and by high concentrations of NaCl. A possible function for this endonuclease in bacterial transformation is discussed.

Bacterial transformation is a process of intercellular transfer of genetic information in which DNA molecules bind to competent recipient cells, penetrate the surface layers of those cells, and recombine with the recipient genome. In the process of uptake and penetration, high-molecular-weight duplex-transforming DNA attaches to the surface of competent *Bacillus subtilis* cells in an endwise manner and enters in a linear progressive fashion (Davidoff-Abelson and Dubnau, 1973). Competent cells then convert transforming DNA of higher molecular weight to double-stranded fragments which possess lecular weights of about 9×10^6. These fragments are rated by endonucleolytic cleavage in the periplasmic space. These double-stranded fragments are then converted to single-stranded fragments, probably during transfer across the membrane.

The fragmentation of DNA in the initial phases of transformation thus suggests the involvement of at least several nucleases. A number of deoxyribonucleases have been demonstrated in *B. subtilis* (Chestukhin and Shemyakin, 1972; Ciarrocchi et al., 1976; Hayase et al., 1975; Kageyama, 1970; Kanamori et al., 1974a, 1974b). Two endonucleases have been suggested as candidates for the double-stranded fragment generating enzyme. One is the manganese-stimulated endonuclease described by Scher and Dubnau (1973, 1976), and the other is the magnesium-dependent heat-activated nuclease first demonstrated by McCarthy and Nester (1969).

In an effort to characterize the magnesium-dependent endonuclease, a purification technique was developed for the

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isolation of the enzyme from a competent strain of *B. subtilis*. The protein was found to have a molecular weight of approximately 31 000 daltons and to be composed of two subunits. Both native and single-stranded DNA are hydrolyzed by the nuclease, although the native form is the preferred substrate. We have also found that the enzyme can be activated at least 10- to 15-fold by heat treatment or treatment with 3 M guanidine hydrochloride.

Experimental Procedure

Materials

Biochemicals. Streptomycin sulfate, calf thymus DNA, sodium dodecyl sulfate, deoxyribonuclease I, subtilisin, lysozyme, bovine serum albumin (fraction V), *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, phenylmethanesulfonyl fluoride, and all of the protein standards used as molecular-weight markers were purchased from Sigma Chemical Co. Cellex D, Cellex P, acrylamide (electrophoresis purity), and *N,N*-methylenebisacrylamide were from Bio-Rad Laboratories. *N,N,N′,N′*-tetramethylethylenediamine and ethylmethanesulfonate were obtained from Eastman. Agarose was purchased from Marine Colloids Inc., Proteinase K was from EM Laboratories, Inc., diethylaminoethylcellulose paper (DE 81) was from Reeve Angel, yeast extract was from Difco, acid-hydrolyzed casein was from Nutritional Biochemicals Corp., and guanidine hydrochloride was from Schwarz/Mann. Ribonuclease A, venom phosphodiesterase, and spleen phosphodiesterase were obtained from Worthington Biochemical Corp. [^{3}H]thymine was a product of New England Nuclear Corp. Nitrocellulose membrane filters (type SM 11306, 25 mm) were obtained from Sartorious-Membranfilter GmbH.

Bacterial Strains. The following strains were used in the course of this study: *Bacillus subtilis* AC-1, 168, SR22, and SCR114; *B. licheniformis* ATCC 8480; *B. amyloliquefaciens* H; *B. pumilus* ATCC 7065; and *Escherichia coli* W3110. *B. subtilis* phages ϕ1, ϕ29, ϕe, and ϕ49 were prepared as described by Kawamura and Ito (1974).

Methods

Determination of Protein and Nucleic Acid Content. Protein was estimated by the method of Waddell (1956) using bovine serum albumin as standard. DNA was determined by the method of Saito and Masamune (1964).

Nucleic Acid Preparation. A thymine-requiring strain of *B. subtilis* 168 (*B. subtilis* SCR114, kindly supplied by J. Ito) was grown to late-log phase at 37 °C in Spizizen's minimal salts (Spizizen, 1958) supplemented with 0.5% glucose, 0.05% acid-hydrolyzed casein, and 2 μg/ml of carrier thymine with [^{3}H]thymine to a final concentration of 10 μCi/ml. Unlabeled DNA was prepared by growing cells in antibiotic medium 3 (Difco) to late-log phase at 37 °C.

The cells from 100 ml of culture were suspended in 20 ml of 10 mM Tris-HCl[1] (pH 7.5)–10 mM EDTA and incubated at 37 °C for 30 min in the presence of 100 μg/ml of lysozyme. The suspension was then treated with 100 μg/ml of ribonuclease A for 10 min at 37 °C followed by proteinase K digestion at 100 μg/ml in the presence of 0.1% sodium dodecyl sulfate at 37 °C for 1 h. The lysate was then twice phenol extracted and dialyzed extensively against SSC buffer (0.15 M sodium chloride–0.015 M sodium citrate).

Viral DNA was prepared by the method of Ito et al. (1976). DNA was denatured by heating at 100 °C for 10 min and immediately cooling in ice. Ribosomal RNA was prepared by the method of Oishi and Sueoka (1965).

Endonuclease Assay. Endonucleolytic deoxyribonuclease activity was assayed as described by Geiduschek and Daniels (1965). The standard reaction mixture contained in 255 μl: 100 mM Tris-HCl (pH 8.1), 20 mM $MgCl_2$, and 7.5 μg of [^{3}H]-DNA. The reaction was initiated by the addition of 5 μl of enzyme and terminated after 1 min at 25 °C with the addition of 200 μl of 0.2 N NaOH. After 15 min in ice, 1.5 ml of 0.1 M NaH_2PO_4 was added. The mixture was filtered through nitrocellulose filters as described by Geiduschek and Daniels (1965). The filters were dried and counted in a toluene-based scintillation fluid. Controls of DNA not treated with enzyme were included in each assay to determine the maximal radioactivity retainable on a filter. All assays were done in triplicate. Activity was expressed either as the percent counts lost from the filter compared with a no-enzyme control or in units. A unit of activity is defined as the amount of enzyme that makes 0.1 μg of [^{3}H]DNA filterable after 1 min at 25 °C. Specific activity is expressed as units per milligram of protein.

Exonuclease Assays. Exonucleolytic deoxyribonuclease activity was measured by determining the release of acid-soluble material from radioactive DNA. The reaction mixture contained in 150 μl: 100 mM Tris-HCl (pH 8.1), 20 mM $MgCl_2$, and 12 μg of [^{3}H]DNA. The reaction was initiated by the addition of purified endonuclease (550 units) and terminated after 30 min at 37 °C by the addition of 0.2 ml of 50% trichloroacetic acid and 0.2 ml of 2.5 mg/ml of calf thymus DNA. The samples were centrifuged at 400*g* for 10 min at 20 °C following a 20 min incubation at 0 °C. Aliquots of 0.1 ml were dried on filter paper disks and measured for radioactivity.

Ribonuclease Assay. Ribonuclease activity was determined by two methods. One procedure measured the release of acid-soluble material from radioactive ribosomal RNA. The reaction mixture contained in 150 μl: 100 mM Tris-HCl (pH 8.1), 20 mM $MgCl_2$, and 3.9 μg of [^{3}H]RNA. The reaction was initiated by the addition of purified nuclease (550 units) or 25 μg of ribonuclease A and terminated after 30 min at 37 °C by the addition of 0.2 ml of 50% trichloroacetic acid and 0.2 ml of 2 mg/ml of bovine serum albumin. The samples were centrifuged at 400*g* for 10 min at 20 °C following a 20-min incubation at 0 °C. Aliquots of 0.1 ml were dried on filter paper disks and measured for radioactivity.

In the second method, aliquots of the reaction mixtures described for the first method were applied to 5–20% sucrose gradients following incubation with the nucleases.

Sucrose Gradient Centrifugation. [^{3}H]DNA products of enzyme hydrolysis were sedimented through 5–20% linear sucrose gradients in 0.05 M Tris-HCl (pH 7.4)–0.1 M NaCl–0.01 M EDTA–0.1% sodium dodecyl sulfate in a SW56 rotor at 48 000 rpm for 90 min at 20 °C. After centrifugation, 10-drop fractions were collected on glass filters. The filters were dried, and radioactivity was determined. ^{14}C-labeled ribosomal RNA's were used as markers.

Preparation of Crude Extracts for Molecular Weight Determination. Crude extracts were prepared by sonication of *B. subtilis* cells suspended in sonication buffer (100 mM Tris-HCl–10 mM EDTA–1 mM phenylmethanesulfonyl fluoride, pH 7.5) at 0 °C for 12 min. The sonicates were centrifuged at 37 000*g* for 30 min. Protein concentrations in the resulting extracts were usually 40–60 mg/ml.

Polyacrylamide Gel Electrophoresis. Analytical disc gel electrophoresis was performed as described by Reisfeld et al.

[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl.

(1962) using the catalyst system of Jordan and Raymond (1969). The acrylamide gel concentration was varied from 8 to 20%, and the electrophoresis was carried out at 25 °C using a constant current of 4 mA/gel. The gels were stained for 2 h in a 0.5% solution of amido black in 7% acetic acid.

Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (1969). Electrophoresis was conducted for 5 h at 3 mA/gel. Staining was accomplished with Coomassie brilliant blue.

Agarose Gel Electrophoresis. Electrophoresis of DNA in 1% Agarose gels was performed as described by Ito et al. (1976).

Analysis of the Termini of the Degradation Products. 17.4 μg of [^{3}H]DNA was incubated with 1650 units of purified endonuclease in 100 mM Tris-HCl (pH 8.1)–20 mM $MgCl_2$ in a total volume of 300 μl. After 15 min of incubation at 25 °C, the reaction was stopped by making the solution 33 mM in EDTA and heating for 10 min at 100 °C. A control tube containing only DNA and reaction mixture was treated identically. To 75-μl aliquots of the control and hydrolyzed DNA the following were added: (a) 15 μl of 250 mM Tris-HCl (pH 8.0)–50 mM $MgCl_2$, (b) 15 μl of 2 mg/ml of venom phosphodiesterase in 250 mM Tris-HCl (pH 8.0)–50 mM $MgCl_2$ and 2.4 μl of 1 M $MgCl_2$, (c) 15 μl of 2 mg/ml of spleen phosphodiesterase in 250 mM Tris-HCl (pH 8.0). These mixtures were incubated at 37 °C for 4 h. Following incubation, the samples were analyzed by descending chromatography on Whatman DEAE-cellulose (DE 81) paper with 0.3 M ammonium formate (pH 6.1).

Molecular-Weight Determinations. Determination of molecular weight by polyacrylamide gel electrophoresis was performed according to Hedrick and Smith (1968), except that the pH 4.5 system of Reisfeld et al. (1962) was used. Separating gels of 8, 10, 12, 14, 16, 18, and 20% acrylamide were employed.

Gel permeation column chromatography was performed with a 1.5 × 90 cm column packed with Sephadex G-150 equilibrated in 0.1 M Tris-HCl (pH 7.5)–0.02% sodium azide. The column was pumped at a constant flow rate of 12.4 ml/h. The molecular weight of the nuclease was determined by the method of Andrews (1964).

The data obtained in each procedure were analyzed using the least-squares method and regression equations to determine the molecular-weight estimates.

Mitomycin C Induction of PBSX. B. subtilis AC-1 was grown in Antibiotic Medium No. 3 at 37 °C with shaking. The turbidity was followed by a Klett-Summerson colorimeter (equipped with a no. 66 filter). When the turbidity reached approximately 30 units, the culture was treated with mitomycin C at a concentration of 1 μg/ml for 20 min. The antibiotic was removed by centrifugation at 10 400*g* for 10 min at 4 °C, and the cells were resuspended in the same volume of fresh medium. The culture was incubated for an additional 2 h. At that time, cell debris and unlysed cells were removed by centrifugation at 10 400*g* for 10 min at 4 °C. The lysate was assayed for endonuclease activity before and after a heat treatment at 100 °C for 30 min.

Transformation Procedure. For transformation procedure, the protocol of Anagnostopoulos and Spizizen (1961) was followed. The DNA concentration was 1 μg/ml, and the cells were exposed to DNA for 30 min.

Results

Purification of the Enzyme. All procedures were carried out at 0–4 °C, unless otherwise indicated.

(1) Growth of Bacteria. Cultures of *B. subtilis* AC-1 (Trp, Met, Lys) were grown at 37 °C with vigorous aeration in a 130-l. capacity fermentor. The 100 l. of growth medium contained minimal salts (Spizizen, 1958) supplemented with 0.5% glucose, 0.1% yeast extract, 0.25% acid-hydrolyzed casein, 5 mM $MgSO_4$, and 50 μg/ml each of tryptophan, lysine, and methionine. The cells were harvested in exponential phase by centrifugation, washed with cold sonication buffer, and stored as a frozen paste at −70 °C.

(2) Preparation of Extract. 130 g of frozen cell paste was thawed, suspended in 300 ml of sonication buffer, and disrupted in 70-ml batches at 0 °C with an ultrasonic probe (Branson Instruments Inc.) for 10 min. The resulting sonicate was centrifuged at 37 000*g* for 30 min.

(3) Heat Treatment. The supernatant was divided equally into two 500-ml flasks, boiled for 40 min, and centrifuged at 27 000*g* for 15 min.

(4) Streptomycin Sulfate Fractionation. The supernatant was transferred to an ice bath and 12.7 g of streptomycin sulfate, dissolved in 50 ml of sonication buffer, was added slowly and with stirring. After 1 h, the suspension was centrifuged at 27 000*g* for 10 min.

(5) Ammonium Sulfate Fractionation. The supernatant was dialyzed overnight against an equal volume of saturated ammonium sulfate in 50 mM Tris-HCl–5 mM EDTA. The precipitate was removed by centrifugation at 27 000*g* for 10 min. The resulting supernatant was dialyzed against two changes of 2 l. of DEAE column buffer (10 mM imidazole, pH 6.5, 0.002% Hibitane).

(6) DEAE Column Chromatography. A 2.6 × 30 cm column was prepared with Cellex-D equilibrated in DEAE column buffer. The dialyzed ammonium sulfate fraction was clarified by centrifugation at 27 000*g* for 10 min and applied to the column at a flow rate of 50 ml/h. Fractions of 5.7 ml were collected. The column was washed with DEAE column buffer until the A_{280} of the effluent was below 0.01. The enzyme activity was found to wash through the column. The endonuclease-containing fractions were combined and dialyzed against three changes of 4 l. of phosphocellulose column buffer (5 mM sodium phosphate, pH 7.5–0.02% sodium azide).

(7) Phosphocellulose Column Chromatography. The dialyzed DEAE column fraction was applied to a 2.6 × 25 cm Cellex-P column equilibrated in the phosphocellulose column buffer. The column was washed with the column buffer at a flow rate of 46 ml/h until the A_{280} was below 0.01. Fractions of 5.1 ml were collected. The enzyme was eluted with a linear gradient consisting of 250 ml of phosphocellulose column buffer containing 0.2 M NaCl and 250 ml of phosphocellulose column buffer containing 0.8 M NaCl.

This purification procedure is summarized in Table I. Upon acrylamide gel electrophoresis at pH 4.5, the purified endonuclease was shown to be electrophoretically pure (Figure 1). This preparation was also free of exonuclease activity. The enzyme was purified approximately 750-fold with a recovery of 3%. The subsequent characterizations were performed on the product of four different fractionations using this procedure, giving comparable overall purifications.

Stability and Storage of the Endonuclease. The purified nuclease was stable to reheating to 100 °C for 10 min and maintained its activity without appreciable loss at 4 °C for 1 year. However, the enzymatic activity was rapidly lost upon dilution to protein concentrations below 0.01 mg/ml.

Molecular Weight of the Endonuclease. Molecular weight estimates of the enzyme were made employing two techniques. The method of Hedrick and Smith (1968) utilizes disc gel

TABLE I: Purification of *B. subtilis* Magnesium-Dependent Endonuclease.

Step	Total Protein (mg)	Total Units ($\times 10^{-3}$)	Sp Act. (units/mg)	% Recovery
Crude	20 486	2 163	106	
Heat treatment	6 350	22 134	3 486	100.0
Streptomycin sulfate	2 978	5 415	1 819	24.5
0–50% ammonium sulfate	1 298	5 207	4 012	23.5
DEAE-cellulose	94	1 995	21 080	9.0
Phosphocellulose	8	652	82 370	3.0

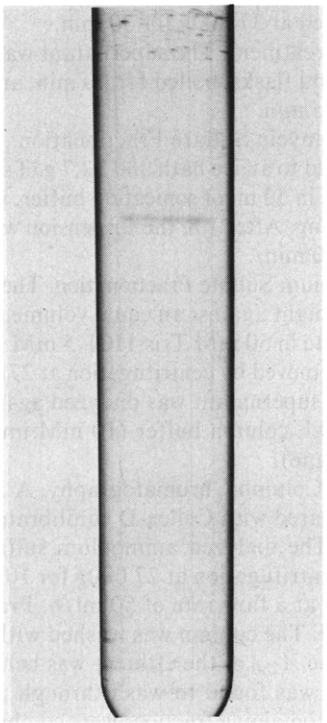

FIGURE 1: Polyacrylamide gel electrophoresis of *B. subtilis* magnesium-dependent endonuclease at pH 4.5. Direction of migration was toward the cathode (bottom).

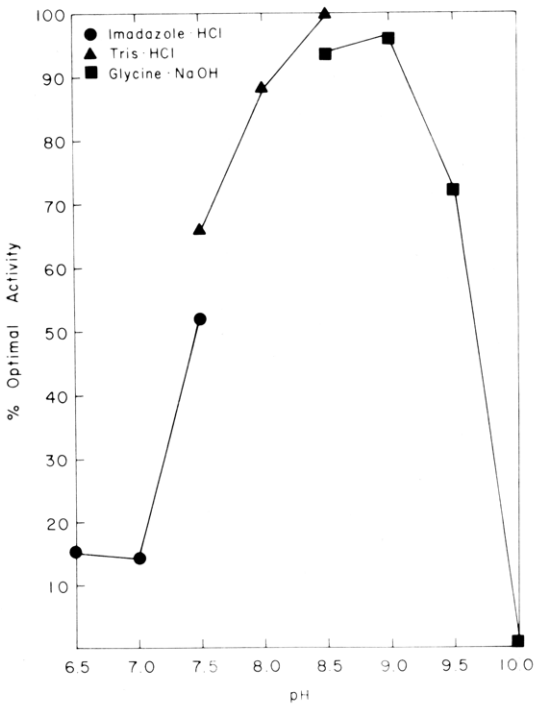

FIGURE 2: The effect of pH on the endonucleolytic activity of the enzyme. Purified enzyme was assayed under standard conditions, except that the pH and buffers were varied as indicated.

electrophoresis at various acrylamide gel concentrations. First, the slope of a plot of the log of protein mobility relative to the dye front vs. acrylamide gel concentration was determined for a series of protein standards and for the endonuclease. The slopes obtained for the standards were plotted against their respective molecular weights. A molecular weight of approximately 29 200 was obtained for the endonuclease.

Because all enzymatic activity was lost when purified nuclease was applied to the Sephadex G-150 column, crude extracts were employed to determine the molecular weight of the protein. Crude, unheated extracts were applied to the Sephadex column. Fractions were collected, boiled for 30 min, and assayed for endonuclease activity. Two peaks of activity were obtained: first, a broad peak ranging from the void volume to a molecular weight of approximately 100 000, and then a sharp peak with a molecular weight of approximately 35 000. This value of 35 000 was obtained by comparing the elution volume of the endonuclease with that of various protein standards.

When the crude extract was heat treated prior to application to the column, a single broad peak was observed with molecular weight ranging from 2 000 000 to 300 000. Dialysis of the heat-activated extract against assay buffer (100 mM Tris-HCl–20 mM $MgCl_2$, pH 8.1) overnight at 25 °C produced no appreciable change in the elution profile.

However, if this dialyzed, heat-activated extract was treated with DNase I prior to application to the column, and then reheated to inactivate the DNase I, a broad peak of nuclease activity with a molecular weight range of 60 000 to 35 000 was observed. These data indicate that the heat-activated nuclease binds to DNA following activation and remains bound following limited cleavage of the DNA.

Subunit Molecular Weight. The purified enzyme was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the method described by Weber and Osborn (1969). The enzyme migrated as a single band in the presence of the denaturing agent. A comparison of the mobility of the nuclease subunits with those of the protein standards indicates a subunit molecular weight of approximately 15 200.

Properties of the Enzyme. The pH dependence of the purified enzyme was evaluated with imidazole hydrochloride, Tris-HCl, and glycine/NaOH buffers. A pH optimum of the purified enzyme ranged from pH 8 to 9 with measurable activity present from pH 7.5 to 9.5 (Figure 2).

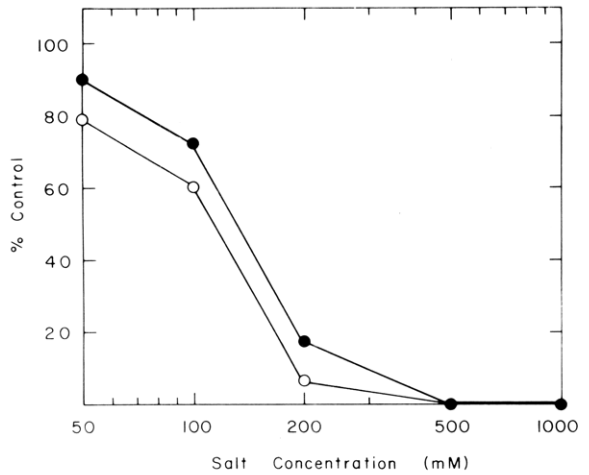

FIGURE 3: Inhibition of endonuclease activity by sodium chloride and guanidine hydrochloride. Purified enzyme was assayed under standard conditions in the presence of various concentrations of sodium chloride (●–●) and guanidine hydrochloride (O–O).

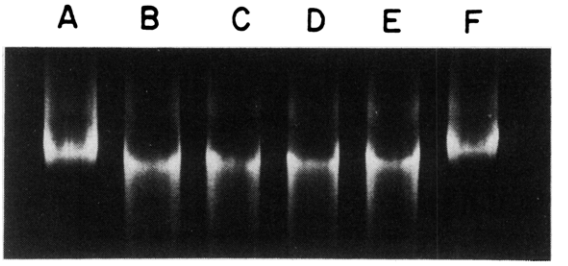

FIGURE 4: Agarose slab gel electrophoresis of endonuclease-treated *B. subtilis* DNA. DNA sample (0.5 μg) was applied to 1% Agarose gels and electrophoresed at a constant current of 30 mA for 5 h at room temperature. Sample slots A and F were untreated controls. Sample slots B, C, D, and E were respectively 10-, 20-, 60-, and 120-min endonuclease digests. The reaction mixture contained 0.7 μg of DNA per unit of endonuclease.

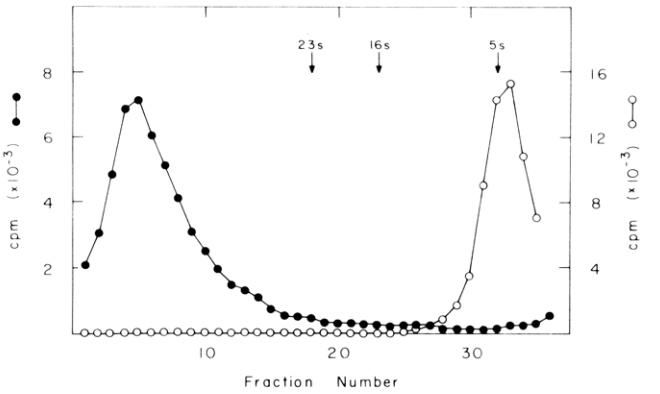

FIGURE 5: Sucrose gradient sedimentation of DNA substrate and product. The DNA was digested as described in the text. (●–●) DNA substrate; (O–O) endonuclease DNA cleavage product.

The optimum magnesium concentration was found to be 20 mM. At magnesium concentrations greater than 30 mM, marked inhibition of the enzyme activity was observed. The magnesium ions could be partially replaced by manganous ions, although concentrations of 100 mM were required to attain activity levels comparable to those obtained with 20 mM magnesium. Calcium was found to be completely ineffective as a cofactor for this nuclease. The enzymatic activity was completely abolished when 10 mM EDTA was substituted for magnesium in the assay buffer.

Sodium phosphate buffer, 50 mM, pH 8.0, used in the assay mixture in place of Tris-HCl, inhibited the activity by 80%. However, the enzymatic activity was not affected by 1 mM *N*-ethylmaleimide or 1 mM *p*-chloromercuribenzoic acid.

The purified endonuclease was sensitive to increasing concentrations of various salts. As shown in Figure 3, complete inhibition of the enzymatic activity was observed when either sodium chloride or guanidine hydrochloride was present in the assay mixture at a concentration of 0.5 M.

Substrate Specificity of the Endonuclease. The substrate specificity of the nuclease was examined using DNA isolated from *B. subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus pumilus,* and *Escherichia coli,* as well as DNA isolated from the *B. subtilis* phages ϕ1 and ϕ29. Following treatment with the heat-activated nuclease, aliquots were subjected to Agarose gel electrophoresis. In all cases, a single band of DNA was observed which migrated more rapidly than the untreated DNA (Figure 4).

Native *B. subtilis* DNA was alkylated and depurinated by the method of Verly and Rassart (1975). No decrease in nuclease activity was observed when the alkylated and depurinated DNA's were employed as substrates. Native *B. subtilis* DNA was also irradiated with ultraviolet light by the method of Levine et al. (1966), producing a maximum of thymine dimers. This dimer-containing DNA was hydrolyzed equally as well as native unirradiated DNA. The enzyme was also capable of hydrolyzing DNA isolated from the *B. subtilis* phages ϕe and ϕ49. The DNA from these viruses contains hydroxymethyluracil in place of thymine.

However, when heat-denatured *B. subtilis* DNA was used as substrate, a slight reduction in the hydrolytic activity of the enzyme was observed. The magnesium-dependent endonuclease hydrolyzed single-stranded DNA at about 80% the rate at which it hydrolyzed double-stranded DNA.

The purified, magnesium-dependent endonuclease was shown to have no ribonuclease activity. *B. subtilis* ribosomal RNA was incubated with purified nuclease under conditions which result in complete degradation of the RNA by ribonuclease A. No change in the sedimentation profile in sucrose gradients of the ribosomal RNA was observed following treatment with the magnesium-dependent nuclease.

Structure of the Termini Produced by Endonuclease Action. In order to determine the structure of the termini produced by the endonuclease, the product of the reaction was incubated separately with two exonucleases: snake venom phosphodiesterase, which requires a free 3′-OH end and produces 5′-mononucleotides, and with spleen phosphodiesterase, which requires a free 5′-OH end and produces 3′-mononucleotides.

Mononucleotides were produced only in the presence of venom phosphodiesterase, indicating that the endonuclease cleaves DNA producing 5′-phosphate and 3′-OH termini.

DNA Product Size. DNA (13 μg) was treated with 550 units of the purified magnesium-dependent endonuclease for 30 min at 25 °C and centrifuged in a sucrose gradient. The untreated DNA had an *S* value of 42–44 S. The smallest double-stranded product obtained from such a substrate had an *S* value of approximately 4 S (Figure 5). No further reduction in the size of the product was observed by doubling the enzyme concentration or increasing the length of incubation to 180 min.

Activation of the Magnesium-Dependent Endonuclease.

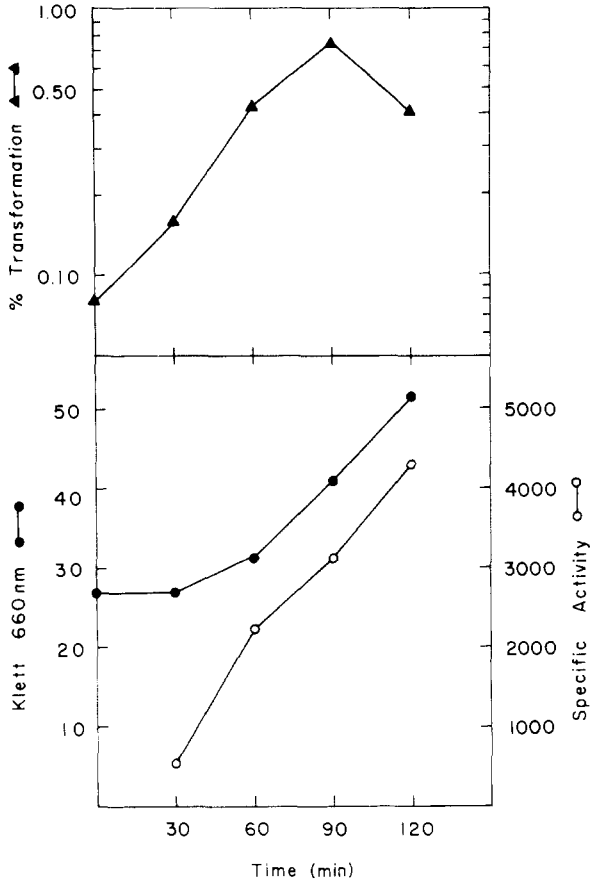

FIGURE 6: Synthesis of heat-activatable endonuclease activity during competence development. The *B. subtilis* AC-1 cells were transformed for the *Trp* marker. Specific activities were determined as described in the text.

As reported by McCarthy and Nester (1969), heating the crude extract to 80, 90, or 100 °C for 30 min activated the enzyme. The heat activation was greatest in the presence of protease inhibitors. Attempts were made to activate the enzyme using other treatments. Extracts for these experiments were prepared in 100 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$. As shown in Table II, treatment with ribonuclease A, pronase, subtilisin, urea, and acid pH under a variety of conditions produced no activation.

However, activation was obtained when crude, unheated extracts containing protease inhibitors (10 mM EDTA–1 mM phenylmethanesulfonyl fluoride) were treated with guanidine hydrochloride (Table II). At a guanidine hydrochloride concentration of 3 M, activation comparable to heat activation was obtained. The apparently lower levels of activation observed at higher concentrations of guanidine hydrochloride were due to the effect of this compound on the assay system. The activation was not reversed by removal of the guanidine hydrochloride by dialysis. Following dialysis, activation comparable to heat activation was seen in extracts activated by guanidine hydrochloride concentrations from 3 to 6 M.

In order to determine if the enzyme was activated in vivo during PBSX induction, lysates were examined for endonuclease activity. Endonuclease activity was observed in mitomycin C induced lysates of *B. subtilis* AC-1 only following heat treatment. Therefore, this magnesium-dependent endonuclease is probably not the nuclease responsible for DNA breakdown following PBSX induction.

Magnesium-Dependent Endonuclease Levels during Competence Development. In order to determine the level of heat-activatable endonuclease in competent cultures, precompetent cells were transferred to transformation medium and periodically assayed for transformability and heat-activatable endonuclease activity. As shown in Figure 6, the cellular level of this nuclease, indicated by the specific activity, increased essentially linearly as the cells became competent. From these data, it can be seen that, under physiological conditions which induced the development of competence, there was a specific synthesis of the heat-activatable nuclease.

TABLE II: Activation Treatments of the Magnesium-Dependent Endonuclease.

Treatment	Temp (°C)	Time (min)	% Heat Activated Act.
Control	0, 37	30	0.0
Heat	100	30	100.0
Ribonuclease (200 μg/ml)	37	30	0.0
Pronase (10 μg/ml)	37	5	0.0
	37	10	0.0
	37	30	0.0
(100 μg/ml)	37	30	0.1
	37	90	0.0
Subtilisin (10 μg/ml)	37	5	0.0
	37	10	0.0
	37	30	0.0
(1 mg/ml)	37	30	0.0
Urea (1.5 M)	37	30	0.0
(4.0 M)	37	30	1.5
pH 3.5	0	30	0.0
Guanidine-HCl (1 M)	37	15	0.0
(2 M)	37	15	36.7
(3 M)	37	15	96.8
(4 M)	37	15	93.0
(5 M)	37	15	86.3
(6 M)	37	15	84.0

Discussion

The procedure described for the purification of the magnesium-dependent endonuclease of *B. subtilis* results in an electrophoretically homogenous enzyme. The loss of activity during isolation may be due in part to nuclease inactivation occurring when the protein concentrations dropped below 10 μg/ml. The use of protease inhibitors in the initial stages of purification greatly increased the recovery of enzymatic activity.

The molecular weight value obtained for this magnesium-dependent endonuclease varied somewhat with the method. A value of 29 200 was obtained by the method of Hedrick and Smith (1968), while 35 000 was observed with Sephadex G-150 chromatography. These values are smaller than the 66 000 value reported by McCarthy and Nester (1969) using sucrose gradient centrifugation. The molecular weights of two other *B. subtilis* endonucleases are alse less than 66 000. The single-stranded DNA-specific enzyme has a molecular weight of approximately 30 000 (Ciarrocchi et al., 1976) and the manganese-stimulated endonuclease has a reported value of 46 000 (Scher and Dubnau, 1976).

The subunit molecular weight of the magnesium-dependent endonuclease was determined to be 15 200, suggesting a dimeric structure for the active enzyme. No information on the subunit structure of the other *B. subtilis* endonucleases is available.

Two main fractions of heat-activatable activity are separated

when unheated extracts were applied to a Sephadex G-150 column. This phenomenon was also observed with this nuclease by McCarthy and Nester (1969) using sucrose gradient centrifugation. If the extract was heat treated prior to column chromatography, a single broad peak of activity was observed having a molecular weight in excess of 300 000. When this heat-treated extract was also treated with DNase I, the molecular weight of the observed activity was reduced to a 35 000 form. These data indicate that the heat-activated nuclease binds to DNA following activation.

The magnesium-dependent enzyme is similar to the manganese-stimulated nuclease and the single-stranded DNA-specific endonuclease in its sensitivity to increasing concentrations of various salts. High concentrations of NaCl greatly inhibited the reaction. When the salt was removed from the enzyme by extensive dialysis, no inhibition of enzymatic activity was seen. This inhibition was observed only when the salt was present in the reaction mixture. This would suggest that the salt does not affect the protein per se but some aspect of the enzymatic hydrolysis.

Unlike the single-stranded DNA-specific endonuclease isolated by Ciarrocchi et al. (1976), the magnesium-dependent nuclease was unaffected by the sulfhydryl group reagents *N*-ethylmaleimide and *p*-chloromercuribenzoic acid. The endonuclease specific for single-stranded DNA hydrolyzed single-stranded DNA at a rate approximately 40 times greater than double-stranded DNA, while the magnesium-dependent enzyme prefers double-stranded DNA. These differences together with different cofactor requirements would indicate that, contrary to the suggestion by Ciarrocchi et al. (1976), the magnesium-dependent nuclease and the single-stranded DNA specific endonuclease are not one and the same enzyme.

The magnesium-dependent endonuclease was able to cleave linear duplex DNA from all sources examined, including homologous DNA, consistent with the activity of an enzyme capable of converting high-molecular-weight DNA to double-stranded fragments. Heat-denatured DNA also served as substrate. The enzymatic activity was not inhibited by the presence of methylated bases, apurinic regions, hydroxymethyluracil, or thymine dimers in native DNA molecules. However, the enzyme was unable to hydrolyze ribosomal RNA and possessed no exonuclease activity. The oligonucleotide products of the hydrolysis are terminated by 5′-phosphate groups similar to the products of pancreatic DNase and *E. coli* endonuclease I (Lehman et al., 1962).

A value of 4 S was obtained for the minimum size of the double-stranded product generated by the action of the magnesium-dependent endonuclease on linear, duplex DNA. This value is in good agreement with the smallest product size of 4.8 S (1.1×10^5 daltons) reported by McCarthy and Nester (1969) for this same nuclease. The reason for the inability of the nuclease to continue degradation of the DNA beyond this point is uncertain. The smaller size of the DNA fragments may render them unsuitable as substrates, or the number of cleavage sites may be limiting. It should be noted that the size of the DNA product in vitro was dependent on the enzyme concentration, rather than length of incubation beyond 5 min. Addition of more enzyme results in further degradation (until the minimum product size is attained). This suggests that the nuclease was in some way inactivated or bound irreversibly in the process of hydrolyzing the DNA. This inactivation process may be similar to that proposed for the *E. coli* B restriction enzyme (Horiuchi et al., 1974).

The mechanism for the activation of this endonuclease has not as yet been determined. The activation produced by heat or guanidine hydrochloride treatment may result from a conformational change in the enzyme and/or from the inactivation or irreversible dissociation of an inhibitor.

Because the extracts of *B. subtilis* contain many nucleases with similar activities, it is difficult to estimate the amount of magnesium-dependent endonuclease which is in the active form at any specific time. It is possible that the intracellular levels of the inactive enzyme reflect the level of the nuclease which exists in the active form.

The presence of an inactive endonuclease in extracts of the transformable *B. subtilis* suggests that the inactivation mechanism, whether inhibitor or specific protein conformation, may function as a protective device for replicating cellular DNA. Possibly the inactivation mechanism operates during the intracellular stage of the nuclease, i.e., following synthesis. Once the enzyme is localized near the periphery or positioned in the cell membrane, the nuclease could then be activated by some cellular mechanism in response to appropriate physiological conditions.

It has been shown that both competent and noncompetent *B. subtilis* 168 cells decrease the biological activity of transforming DNA (Joenje and Venema, 1975) and that this inactivation is primarily due to the formation of double-stranded fragments. The size of these fragments is much larger than the minimum product size generated by the magnesium-dependent endonuclease. However, the size of the cleavage product formed by this enzyme is dependent, at least in vitro, on the relative endonuclease concentration. DNA fragments of the size observed in vivo can be produced in vitro by adjusting the endonuclease concentration relative to the DNA concentration. Since the uptake and transport of DNA by competent cells is a very dynamic process, it could easily be assumed that the endonuclease has access to the donor DNA for only a brief time before the substrate is rendered inaccessible by transport across the membrane. Thus, the fragmentation of transforming DNA could easily be accomplished by this nuclease. Further, the enzyme was demonstrated to be selectively synthesized in competent cultures. Since competence is a highly complex physiological state requiring the coordination of a number of diverse components, the synthesis of the magnesium-dependent endonuclease during competence development may be indicative of its significance as an element in the DNA-processing system.

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Serine Transhydroxymethylase: Evidence for a Sequential Random Mechanism†

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ABSTRACT: Initial velocity patterns in the presence of product and dead-end inhibitors suggest that in reaction 1 the addition of substrates and release of products occur by a sequential random mechanism: L-serine + tetrahydrofolate ⇌ glycine + 5,10-methylenetetrahydrofolate. This interpretation is supported by equilibrium isotope-exchange studies. The relative maximum rates of exchange of L-serine ⇌ glycine and L-serine ⇌ 5,10-methylenetetrahydrofolate in reaction 1 were not inhibited by high levels of substrates. The relative rates of these two exchange reactions were similar but were not identical. These results suggest that the catalytic interconversion and dissociation of substrates are of the same order of magnitude. Reaction 1 represents the transfer of a one-carbon group from the third carbon of L-serine to tetrahydrofolate. Inhibition studies showed that abortive enzyme ternary complexes are formed with L-serine and tetrahydrofolate compounds, which also contain a one-carbon group, e.g., 5-methyltetrahydrofolate and 5,10-methylenetetrahydrofolate. This suggests that the one-carbon binding site can accommodate two one-carbon groups simultaneously without serious steric hindrance.

Serine transhydroxymethylase (EC 2.1.2.1) catalyzes the following reactions at rates which are of physiological importance (Wilson and Snell, 1962; Schirch and Mason, 1963; Schirch and Gross, 1968; Palekar, et al., 1973; Ulevitch and Kallen, 1973). H_4-folate[1] is required only for reactions 1 and 2.

L-serine + H_4-folate ⇌ glycine + 5,10-CH_2-H_4-folate (1)

α-methyl-L-serine + H_4-folate ⇌ D-alanine + 5,10-CH_2-H_4-folate (2)

allothreonine ⇌ glycine + acetaldehyde (3)

L-threonine ⇌ glycine + acetaldehyde (4)

β-phenylserine ⇌ glycine + benzaldehyde (5)

aminomalonate ⇌ glycine + CO_2 (6)

In our previous work, we have been primarily concerned with substrate and reaction specificity and with identifying enzyme–substrate complexes (Schirch and Gross, 1968; Schirch and Jenkins, 1964a,b; Schirch and Diller, 1971; Schirch, 1975; Chen and Schirch, 1973b). In this paper, we explore further the properties of reaction 1 with emphasis on the role of H_4-folate.

Enzyme-bound pyridoxal phosphate is a required cofactor for all of the reactions listed. Considerable evidence has been accumulated to show that pyridoxal phosphate in these reac-

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[1] Abbreviations used are: H_4-folate, *dl*-tetrahydrofolate; 5,10-CH_2-H_4-folate, 5,10-methylenetetrahydrofolate; 5-CH_3-H_4-folate, 5-methyltetrahydrofolate; 5-CHO-H_4-folate, 5-formyltetrahydrofolate; 10-CHO-H_4-folate, 10-formyltetrahydrofolate; 5,10-CH^+-H_4-folate, 5,10-methenyltetrahydrofolate; H_2-folate, dihydrofolate; E, serine transhydroxymethylase; NADH, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.