Purification and Properties of the Endonuclease Specific for Apurinic Sites of Bacillus stearothermophilus*

(Received for publication, March 29, 1977, and in revised form, July 21, 1977)

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An endonuclease specific for apurinic sites when double-stranded DNA is used as substrate has been isolated from the thermophilic bacterium, Bacillus stearothermophilus; it is a monomeric protein of about 28,000 daltons, without action on normal DNA strands or on alkylated sites. The enzyme is quite thermostable and in the presence of other proteins, has a temperature of 60°, needs monovalent cations for optimal activity, is insensitive to EDTA, and is inhibited by divalent cations; it has no associated exonuclease activity. These latter properties are closer to those of Escherichia coli thermostable endonuclease IV, which is also insensitive to EDTA and has no exonuclease activity, and very different from those of the main endonuclease for apurinic sites of the same bacterium. The B. stearothermophilus enzyme is more resistant to urea and triphosphates, ligase and its coenzyme (12). Gossard and it resists heating at 45°, is not inhibited by EDTA, and is devoid of exonuclease activity.

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DNA spontaneously loses purines (1) and pyrimidines (2). The rate of base loss is considerably increased by treatment with chemicals like alkylating agents or by exposure to ionizing radiation. Endonucleases specific for apurinic sites in DNA have been found in Escherichia coli (3-5), in animals (6, 7), and in plants (8).

E. coli possesses two endonucleases for apurinic sites. The main enzyme, which is responsible for 90% of the cell activity, has been completely purified by Verly and Rassart (9); it is thermostable, is inhibited by EDTA, needs magnesium ions to be active, and might be the same enzyme as exonuclease III (10, 11). The accessory enzyme, which is responsible for 10% of the cell activity, has been called endonuclease IV (5); it resists heating at 45°, is not inhibited by EDTA, and is devoid of exonuclease activity.

Depurinated DNA has been repaired in vitro with three enzymes: the main E. coli endonuclease specific for apurinic sites, DNA polymerase I and the four deoxyribonucleoside triphosphates, ligase and its coenzyme (12). Gossard and Verly (11) gave the details of the repair molecular mechanism. The spontaneous loss of DNA bases must be very high in thermophilic bacteria at the temperature at which they usually live so that we looked for an endonuclease hydrolyzing a phosphoester bond near apurinic sites in Bacillus stearothermophilus. The enzyme was found and purified. It is thermostable when protected by other proteins; the presence of a high percentage of hydrophobic amino acids, likely grouped in a central core, might explain this property. The B. stearothermophilus enzyme behaves more like endonuclease IV than like the main endonuclease for apurinic sites of E. coli (endonuclease VI).

†† The abbreviation used is: Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
††† This work was supported by grants from the Medical Research Council of Canada and the Fonds de la Recherche Fondamentale Collective of Belgium. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
soluble. The specific radioactivities of these labeled DNAs are adjusted to 60,000 dpm/µg; for the enzymatic reactions, they usually are in Hepes buffer at a concentration of 50 µg/ml.

Labeled T7 phage DNA is prepared according to the method of Crine and Verly (15); it has a specific radioactivity of 150,000 dpm/µg and contains an average of 0.9 break/strand. It is kept at 4 °C in SSC, 0.01 M MgCl₂.

To make alkylated T7 DNA, 0.1 ml of SSC containing 25 µg of T7 [³H]DNA and 0.9 ml of 0.2 M sodium phosphate buffer, pH 7.0, are mixed and methyl methanesulfonate is added to have a 0.02 M final concentration; after 2 h at 37 °C, the solution is dialyzed three times against 500 ml of SSC, 0.01 M MgCl₂. This alkylated T7 [³H]DNA contains approximately 350 methylated sites/strand (16).

To make depurinated T7 DNA, 0.9 ml of 0.01 M acetic acid is added to 0.1 ml of SSC containing 25 µg of T7 [³H]DNA (final pH 4.0); after an incubation of 30 min at 37 °C, the solution is neutralized with 0.1 ml of 1 M sodium phosphate, pH 7.0, and dialyzed three times against 500 ml of SSC, 0.01 M MgCl₂ (15). This depurinated T7 [³H]DNA contains about 30 apurinic sites/strand.

Enzyme Assay—To 20 µl of alkylated-depurinated [³H]DNA (1 µg; 0.05 M Hepes buffer, pH 7.0) are added 20 µl of enzyme solution in the same buffer. After 10 min at 37 °C, the tubes are cooled in crushed ice, 100 µl of SSC containing 200 µg of calf thymus DNA and 900 µl of 5.78% perchloric acid are successively added. After shaking, the tubes are kept 15 min at 0 °C, centrifuged at 12,000 × g for 15 min, and the supernatants are assayed for radioactivity. Controls without enzyme, which are always below 1% of the substrate radioactivity, are subtracted from the experimental values. The corrected results are expressed as fractions of the substrate radioactivity (acid-soluble fraction).

As long as it does not exceed 0.1, the acid-soluble fraction is approximately proportional to the amount of enzyme. The enzyme unit is the activity necessary to give an acid-soluble fraction of 0.1 in the assay.

NaOH Treatment of DNA—To 1 volume of DNA solution is added 1 volume of 0.4 M NaOH; after 15 min at 37 °C, the mixture is neutralized with 1 volume of 0.4 M HCl. This treatment hydrolyzes a phosphoester bond near each apurinic site (4).

Denaturation of T7 DNA by NaOH or Formamide and Estimation of Average Number of Breaks/Strand—After denaturation, DNA is sedimented on neutral sucrose gradients (12). Alkaline treatment hydrolyzes a phosphoester bond near each apurinic site (17), whereas formamide denaturation at neutral pH leaves them intact (18). Consequently, the sedimentation profile after formamide denaturation yields the average number of breaks/T7 DNA strand; after NaOH denaturation, it yields the sum of breaks and intact apurinic sites. The difference between these values gives the average number of intact apurinic sites (i.e. not associated with breaks).

Purification of Enzyme—An 8% streptomycin solution in Buffer I is added dropwise, with constant stirring, to the crude extract from 50 g of bacteria (Preparation I = 440 ml; 44,000 enzyme units; 8 units/mg of protein) at 0 °C to reach a 0.8% final concentration. After mixing for an additional 15 min, the suspension is centrifuged for 30 min at 16,000 × g; the supernatant (Preparation II) is used for further purification.

To Preparation II (505 ml; 43,000 enzyme units; 11 units/mg of protein), 4.0 M NaCl linear gradient in Buffer II; fractions of 10 ml are collected. This procedure results in a specific activity of 23,000 enzyme units with a specific activity of 290 units/mg of protein (Preparation III).

Preparation III is applied on a DEAE-cellulose (Whatman) column (5 × 15 cm) equilibrated with Buffer II (pH 8.0) at a flow rate of 90 ml/h. The column is washed with 75 ml of Buffer II containing 0.05 M NaCl, then eluted, always at the same flow rate, with 2 liters of a 0.05 to 0.25 M NaCl linear gradient in Buffer II; fractions of 15 ml are collected. Fig. 1A shows that two peaks of enzyme activity are eluted between 0.17 and 0.21 M NaCl. Fractions 104 to 116, corresponding to the second peak, are pooled; they contain 23,000 enzyme units with a specific activity of 290 units/mg of protein (Preparation IV).

Preparation IV (186 ml) is dialyzed against Buffer III, then applied, at a rate of 55 ml/h, on a DEAE-Sephadex A-25 column (2.6 × 40 cm) equilibrated with the same buffer (pH 7.5). The column is washed with 100 ml of buffer containing 0.1 M NaCl and the elution is carried out, at the same flow rate, with 1 liter of a 0.1 to 0.2 M NaCl linear gradient in Buffer III; fractions of 10 ml are collected. Fig. 1B shows two peaks of activity; Fractions 66 to 75, corresponding to the first peak, are pooled (Preparation V); they contain 11,000 enzyme units with a specific activity of 1,250 units/mg of protein.

To concentrate Preparation V, the 200 ml are dialyzed against Buffer III and poured on a small column (0.9 × 15 cm) of DEAE-cellulose, which is eluted with 0.5 M NaCl in Buffer III; the protein (detected at 280 nm) is found in three 2.5-ml fractions that are pooled. The 7.5 ml are placed on a Sephadex G-75 column (2.6 × 100
al. (211 using bovine serum albumin as standard.

is carried out with the same buffer at a rate of 20 ml/h, and 5-ml

Samples (200 ~1; 2.5 mg of protein) of the crude extract were

measured and also the distance migrated by the protein (Dp). A

The sample (2 mg), dissolved in 2 ml of Buffer III, 0.1

with Coomassie blue, the total length of the gel (Lb) is again

by the bromphenol blue are measured. After fixation and staining

A Sephadex G-75 column (1.6 x 70 cm) is equilibrated with Buffer

III, 0.1 M NaCl; the void volume (V,) is measured with blue dextran

wheras the total volume (V,) is determined with glycylglycine.

Four different proteins of known molecular weights (ovalbumin,

Sulfate- The method described by Weber and Osbom (20) is followed

in the presence of sodium dodecyl sulfate. Two gels were carried out

using a 2.5% stacking gel (0.5 cm) on top of a 10% separating gel (7

cm). The proteins are denatured, at room temperature or 100º, in

0.1 M Tris-HCl, pH 6.8, containing 1% sodium dodecyl sulfate and

1% 2-mercaptoethanol. After addition of bromphenol blue, aliquots

(100 ~1) containing from 5 to 20 ~g of protein, are placed on the

stacking gels and the electrophoresis is carried out in 0.05 M Tris,

glycine, 0.1% sodium dodecyl sulfate, pH 8.3, buffer at 1.5

mA/tube until the bromphenol blue reaches the end of the gel. The total length (La) of the extracted gel and the distance (Db) migrated by

the bromphenol blue are measured. After fixation and staining with Coomassie blue, the total length of the gel (Lb) is again measured and also the distance migrated by the protein (Dp). A migration coefficient (mc) is then calculated: mc = (Dp x La)/(Db x Lb).

Molecular Weight Estimation by Sephadex G-75 Chromatography—

A Sephadex G-75 column (1.6 x 70 cm) is equilibrated with Buffer

III, 0.1 M NaCl; the void volume (V,) is measured with blue dextran whereas the total volume (V,) is determined with glycylglycine.

Four different proteins of known molecular weights (ovalbumin,

chymotrypsinogen, ribonuclease A, insulin) are used for calibration.

The sample (2 mg), dissolved in 2 ml of Buffer III, 0.1 M NaCl, is

applied to the column and eluted at a rate of 6 ml/h with the same buffer; 3-ml fractions are collected and their absorbance is read at

280 nm to determine the protein elution volume (V,). There is a linear relationship between the logarithm of the molecular weight and the elution constant K, = (V, - V+)/ (V, - V stalk). The optimum pH for the

enzyme activity on alkylated-purinated [3H]DNA (acid-soluble frac-

tion), and the other (B) was stained with Coomassie blue, scanned

at 550 nm, and the percentage of transmittance recorded.

RESULTS

Properties of Bacillus stearothermophilus Crude Extract—

Samples (200 ~1; 2.5 mg of protein) of the crude extract were mixed with 200 ~1 of [3H]DNA (20 ~g/ml), either untreated, alkylated, or alkylated-depurinated, and incubated at 37º; aliquots were taken after 0 to 120 min to measure the acid-soluble radioactivity. Fig. 2 shows that the extract had some action on untreated DNA, more on alkylated DNA, but that its action was far greater when alkylated sites were replaced by apurinic sites (alkylated-depurinated DNA).

Using acetate/barbital buffers (0.14 M sodium acetate, 0.14 M sodium 5,5'-diethybarbiturate), the optimum pH for the

Purification of Bacillus stearothermophilus endonuclease for

apurinic sites—Table I shows that, from the crude extract, a 8100-fold purification is achieved with an overall yield of about 10%.

Polyacrylamide Gel Electrophoresis in Presence of Sodium Dodecyl Suluate—The method described by Weber and Osbom (20) is followed

in the presence of sodium dodecyl sulfate. Two gels were carried out

in parallel. One (A) was cut in 2-mm slices which were assayed for enzyme activity on alkylated-purinated [3H]DNA (acid-soluble frac-

tion), and the other (B) was stained with Coomassie blue, scanned

at 550 nm, and the percentage of transmittance recorded.

Table I

Purification of Bacillus stearothermophilus endonuclease for

apurinic sites

| Preparation     | Volume | Endonuclease | Specific activity |
|-----------------|--------|--------------|------------------|
|                 | ml     | units        | units/mg protein |
| I. Crude extract| 440    | 44,000       | 8                |
| II. Streptomycin | 505    | 43,000       | 11               |
| III. Ammonium sulfate | 100 | 27,000       | 26               |
| IV. DEAE-cellulose | 195 | 23,000       | 290              |
| V. DEAE-Sephadex | 200    | 11,000       | 1,250            |
| VI. Sephadex G 75 | 30     | 9,000        | 5,600            |
| VII. DNA-Sephadex | 3      | 4,200        | 65,000           |

FIG. 2. Action of the crude extract of Bacillus stearothermophilus cells on untreated, alkylated, and alkylated-depurinated DNA. Samples (200 ~1) of the crude extract in Buffer I were mixed with 200 ~1 of 0.05 M HEPES, 0.05 M NaCl, 1 mM MgCl, 7.5 pH buffer containing 4 ~g of [3H]DNA, either untreated (x), alkylated (O), or alkylated-purinated (•); the mixtures were incubated at 37º and aliquots taken from 0 to 120 min to measure the acid-soluble radioactivity. The results were corrected for controls with Buffer I instead of the crude extract; they are expressed as fractions of the substrate radioactivity (acid-soluble fraction).

FIG. 3. Polyacrylamide gel electrophoresis of the purified enzyme in the presence of sodium dodecyl sulfate. Two gels were carried out in parallel. One (A) was cut in 2-mm slices which were assayed for enzyme activity on alkylated-purinated [3H]DNA (acid-soluble fraction), and the other (B) was stained with Coomassie blue, scanned at 550 nm, and the percentage of transmittance recorded.
TABLE II
Amino acid composition of two bacterial endonucleases for apurinic sites

The Escherichia coli results are from Verly and Rassart (9). Because no data were available for cysteine, methionine, and tryptophan, the molar percentages do not take account of the possible presence of these amino acids in the proteins. The polarity index is calculated by summing the polar amino acids and half the total of those of the intermediate class (23).

| Class and amino acid | Bacillus stearothermophilus | Escherichia coli main enzyme |
|----------------------|-----------------------------|-----------------------------|
|                     | molar %                     | molar %                     |
| Polar                |                             |                             |
| Arginine             | 0.0                         | 3.5                         |
| Aspartic acid        | 15.8                        | 11.3                        |
| Glutamic acid        | 4.4                         | 15.1                        |
| Lysine               | 10.0                        | 5.6                         |
| Total                | 30.2                        | 35.5                        |
| Intermediate         |                             |                             |
| Glycine              | 11.5                        | 16.9                        |
| Histidine            | 3.5                         | 1.8                         |
| Serine               | 6.9                         | 13.0                        |
| Threonine            | 6.0                         | 4.5                         |
| Tyrosine             | 0.0                         | 0.9                         |
| Total                | 27.9                        | 37.2                        |
| Nonpolar             |                             |                             |
| Alanine              | 11.0                        | 7.6                         |
| Isoleucine           | 5.7                         | 2.8                         |
| Leucine              | 7.8                         | 5.4                         |
| Phenylalanine        | 2.0                         | 3.9                         |
| Proline              | 8.9                         | 3.5                         |
| Valine               | 8.3                         | 4.4                         |
| Total                | 41.7                        | 27.6                        |

TABLE III
Action of Preparation VI on untreated, alkylated, and depurinated T7 DNA

Samples of 100 µl of Preparation VI (16 enzyme units) in Hepes buffer were incubated for 60 min at 37°C with 100 µl of the same buffer containing 2 µg of T7 [3H]DNA. Controls without enzyme were carried out in the same way. At the end of the incubation, 800 µl of 0.15 M NaCl, 0.015 M EDTA, pH 7.0, were added and the solution was dialyzed at 4°C against the same buffer. Each sample was then split in two parts; one was denatured with NaOH and the other with formamide before sedimentation on neutral sucrose gradients. The average number of breaks/strand was estimated from the sedimentation profile.

| T7 [3H]DNA | Enzyme | NaOH | Formamide | Alkali-labile sites |
|------------|--------|------|-----------|---------------------|
|            |        | breaks/strand |            |                     |
| Untreated  | -      | 2.1  | 2.6       | <0                  |
|            | +      | 2.3  | 2.3       | 0                   |
| Alkylated  | -      | 12   | 7         | 5                   |
|            | +      | 13   | 13        | 0                   |
| Depurinated| -      | 33   | 7         | 26                  |
|            | +      | 30   | 36        | <0                  |

crude extract activity on alkylated-depurinated DNA was found to be at 7.5. Among different buffers at pH 7.5 that were tested, 0.05 M Hepes was found the best for the enzyme activity.

Aliquots of crude extract diluted 100-fold with Buffer I containing 2% bovine serum albumin (20 µl) were incubated with 20 µl of the alkylated-depurinated [3H]DNA solution for 10 min. The optimal temperature for the enzyme was found to be 60°C. Below this temperature, the logarithm of the reaction velocity plotted against 1/T (T = absolute temperature in K) yields a straight line from which an activation energy of 21,000 cal/mol can be calculated for the enzyme-catalyzed reaction.

Physical Properties of Purified Enzyme - Two aliquots of Preparation VII (200 µl), containing 4 µg of protein, were submitted to polyacrylamide gel electrophoresis (see "Materials and Methods"). One of the gels was stained with Coomassie blue; the other was cut in 2-mm slices which were ground in their migration coefficients; Preparation VII gave a single protein band corresponding to the enzyme activity (Fig. 3). In another experiment, the solution of Preparation VII, which contained 1% sodium dodecyl sulfate, and three proteins of known molecular weights (ovalbumin, chymotrypsinogen, ribonuclease A) dissolved in the same buffer, were heated at 100°C for 2 min and submitted to gel electrophoresis. After staining with Coomassie blue, the migration coefficients were calculated.

The molecular weight of the endonuclease was also determined by Sephadex G-75 filtration. Preparation VI was dialyzed against Buffer III and 0.1 M NaCl, and a 2-ml aliquot was filtered through the calibrated Sephadex G-75 column (see "Materials and Methods"). Reference to the calibration curve given by the standard proteins indicated a molecular weight around 27,000.

Preparation VII (180 µg of protein) was dialyzed against water, lyophilized, hydrolyzed in HCl, and analyzed for amino acid content on an automatic JEOL JLC-ASH apparatus. The molar percentages were calculated from the specific absorption determined experimentally with pure amino acids (Table II).

Substrate Specificity of Purified Enzyme - Preparation VI (16 enzyme units) was incubated with 2 µg of T7 [3H]DNA, either untreated, alkylated, or depurinated. Each sample was then split in two parts; one was denatured with NaOH and the other with formamide before sedimentation on sucrose gradients. Table III indicates that the untreated DNA contained no alkali-labile sites (= apurinic sites), the alkylated

TABLE IV
Effect of divalent cations on activity of endonuclease for apurinic sites of Bacillus stearothermophilus

Preparation VI and the alkylated-depurinated [3H]DNA solution were dialyzed three times against 0.06 M Tris HCl, 0.05 M NaCl, 10 mM EDTA, pH 7.5, then three times against 0.05 M Hepes, 0.05 M NaCl, pH 7.5. To 10 µl of enzyme preparation and 20 µl of substrate solution, were added 30 µl of the same Hepes buffer containing the amount of the chloride salt of the divalent cation needed to reach the indicated concentration. After a 30-min incubation at 37°C, the acid-soluble radioactivity was measured; it is expressed in percentage of the value obtained without the divalent cation.

| Cation  | 0.1 mM | 0.5 mM | 2 mM | 5 mM | 10 mM |
|---------|--------|--------|------|------|-------|
| Mg²⁺    | 103    | 89     | 61   | 54   | 43    |
| Ca²⁺    | 95     | 77     | 57   | 66   | 61    |
| CsCl    | 83     | 84     | 74   | 64   | 74    |
| Mn²⁺    | 88     | 92     | 77   | 71   | 63    |
| Cu²⁺    | 79     | 92     | 94   | 83   | 10    |
| Co²⁺    | 109    | 107    | 62   | 12   | 14    |
| Zn²⁺    | 95     | 96     | 77   | 22   | 14    |

* The incubation medium contained 10 mM MgCl₂ plus the indicated concentration of CaCl₂.
An endonuclease which hydrolyzes DNA containing apurinic sites has been purified from Bacillus stearothermophilus cells. The final product appeared as a single protein band in a polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate. The molecular weights of the native enzyme measured on Sephadex G-75 and of the sodium dodecyl sulfate-denatured enzyme determined by gel electrophoresis were nearly the same, the enzyme thus appears to be a monomeric protein of about 28,000 daltons.

With double-stranded DNA, the pure B. stearothermophilus enzyme is strictly specific for apurinic sites. This was shown using T7 phage DNA labeled with tritium and, after denaturation, the sucrose gradient centrifugation technique. Table III shows that the enzyme introduced no break in untreated DNA, and that the action on alkylated DNA was restricted to the alkali-labile sites. Because NaOH does not produce breaks near alkylated sites (22), the conclusion is that the enzyme has no action on alkylated sites, either of the endonuclease type (see results after denaturation with formamide) or of the N-glycosidase type (see results after NaOH denaturation). The pure enzyme did not degrade sonicated DNA or DNA nicked with pancreatic deoxyribonuclease; it is thus without an activity similar to that of Escherichia coli exonuclease III.

The activity of the endonuclease of B. stearothermophilus on apurinic sites is considerably decreased when the DNA is denatured.

Because of its thermoresistance, absence of inhibition by EDTA, and absence of associated exonuclease activity, the endonuclease specific for apurinic sites of B. stearothermophilus resembles more closely the endonuclease IV of E. coli (5) than the main endonuclease specific for apurinic sites found...
in this bacterium by Verly and Paquette (3, 4) and purified by Verly and Rassart (9) (endonuclease VI).

To understand the causes of the heat resistance of the B. stearothermophilus enzyme, we compared the enzyme of this thermophilic bacterium to the thermoceptive E. coli main endonuclease for apurinic sites. Fig. 4 shows that the E. coli enzyme is more readily denatured by urea and detergents. An amino acid analysis revealed a higher percentage of hydrophobic amino acids in the B. stearothermophilus endonuclease (Table II); the polarity index, calculated according to Vanderkooi and Capaldi (23), is 54.1% for the E. coli enzyme and only 44.2% for that of the thermophilic bacterium. Possibly a more important hydrophobic core might be responsible for the higher resistance to denaturation of the B. stearothermophilus endonuclease for apurinic sites.

Acknowledgment—We wish to thank Miss Michéle Paris for her skilled assistance.

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Purification and properties of the endonuclease specific for apurinic sites of 
Bacillus stearothermophilus.
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*J. Biol. Chem.* 1978, 253:850-855.

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