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DNA-DEPENDENT ATPase II FROM *BACILLUS CEREUS*

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Summary

A new DNA-dependent ATPase has been purified close to homogeneity from soluble extracts of *Bacillus cereus*. This enzyme, called ATPase II catalyses the hydrolysis of ATP in the presence of Mg^{2+} or Ca^{2+} and DNA. Single-stranded linear DNA is a cofactor about 3-fold more effective than double-stranded DNA. The enzyme catalyses the strand separation of duplex DNA in the presence of ATP. However, at concentrations higher than 0.5 mM, phosphohydrolysis can occur without concomitant DNA unwinding. The enzyme has a molecular weight of 84 000 according to SDS-polyacrylamide gel electrophoresis. ATPase II is inhibited by adenosine 5′-(β,γ-imido)-diphosphate, actinomycin D and ethidium bromide, but not by nalidixic acid.

Introduction

Some DNA-dependent ATPases of *Escherichia coli* are known to be involved in strand-separation processes including DNA recombination [1,2], and DNA replication [3,4]. However, the biological function of others is unknown [5,6]. DNA-dependent ATPase I of *E. coli* [7] has been shown to be identical with rep gene product [8] and with helicase II [9]. On the other hand, it has been reported that both helicase I and II differ from rep protein of *E. coli* [10]. *E. coli* ATPase II stimulated by double-stranded DNA differs from all other DNA-dependent ATPases which require single-stranded DNA as cofactor [6]. With regard to the growing number and to the similar properties of *E. coli* ATPases it is rather difficult to distinguish them.

Little is known about bacterial DNA-unwinding ATPases except for the *E. coli* enzymes. The aim of this paper is to describe a new DNA-dependent

ATPase (ATPase II) from *B. cereus*, which was detected during the purification of DNA-dependent ATPase I [11].

Materials and Methods

Composition of the buffers used, buffer A: 20 mM Tris-HCl (pH 7.5)/0.1 mM EDTA/2 mM mercaptoethanol/10% (v/v) glycerol. Buffer B: buffer A plus 30% (v/v) glycerol. Buffer C (saline/sodium citrate): 150 mM NaCl/15 mM sodium citrate (pH 7.0).

[γ-^{32}P]ATP (200 Ci/mol) was prepared according to Post and Sen [12]. The unlabelled ATP analogues, adenosine 5′-(β,γ-imido)-diphosphate and 5′-(β,γ-methylene)-diphosphate were from Boehringer.

Protein was determined by the method of Lowry et al. [13], using crystalline bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn [14]. As marker proteins bovine serum albumin (Serva), glyceraldehyde phosphate dehydrogenase (Boehringer), trypsin and ribonuclease (Calbiochem) were used.

ATP was coupled to AH-Sepharose 4B (Pharmacia) as described by Lamed et al. [15]. [^{3}H]DNA ($7 \cdot 10^3$ cpm per nmol) was isolated from *B. cereus 130 thy*$^-$ as reported earlier [16]. DNA from *E. coli* was prepared by the method of Marmur [17]. T7 DNA was prepared according to Richardson [18]. PM 2 DNA was a gift from P. Medveczky, calf thymus DNA was purchased from Merck, and yeast RNA from Calbiochem. Heat denaturation of DNA was carried out at 100°C for 10 min in buffer C followed by rapid chilling in ice.

DNAase I treatment of DNA. Reaction mixtures for incubations with pancreatic endonuclease I contained in 300 μl final volume, 3 nmol Tris-HCl buffer (pH 7.5)/1.5 nmol $MgCl_2$/48 nmol *E. coli* DNA/3 units of pancreatic deoxyribonuclase (Serva). The reaction mixture was incubated at 35°C for 30 min. Reaction was terminated by heating to 60°C for 10 min. Acid-soluble DNA was removed by dialysis against 200 vols. buffer C. Dialyzed material was used as DNAase I-treated DNA.

S_1 nuclease activity was detected in a reaction mixture of 150 μl containing 10 nmol sodium acetate buffer (pH 4.7), 50 nmol NaCl, 0.3 nmol $ZnSO_4$, 10 nmol [^{3}H]DNA and 0.1 unit of S_1 nuclease (Sigma). The mixture was incubated at 35°C for 10 min. Reaction was terminated by addition of 100 μl of 2 M perchloric acid and 50 μl of 5 mg/ml bovine serum albumin. After centrifugation at $4000 \times g$ for 10 min 100-μl aliquots from the supernatant were pipetted into counting vials and radioactivity was measured.

Micrococcal nuclease activity was assayed in a reaction mixture (150 μl) containing 10 nmol [^{3}H]DNA, 5 μmol Tris-HCl buffer (pH 8.8), 0.5 μmol $CaCl_2$, 30 nmol ATP, 50 μg bovine serum albumin and 5 mU of micrococcal nuclease (Boehringer). The reaction mixture was incubated at 35°C for 10 min. Undigested DNA was precipitated by addition of 100 μl 2 M perchloric acid and 50 μl 5 mg/ml bovine serum albumin. The sample was centrifuged at $4000 \times g$ for 10 min. Radioactivity was determined by 100-μl aliquots.

DNA-dependent ATPase assay was carried out by the measurement of $^{32}P_i$ released from [γ-^{32}P]ATP. The reaction mixture (75 μl) contained 5 nmol denatured T7 DNA, 1 nmol [γ-^{32}P]ATP ($1—4 \cdot 10^5$ cpm per nmol), 75 nmol

unlabelled ATP, 75 nmol $MgCl_2$, 750 nmol Tris-HCl buffer (pH 8.0) and enzyme. After incubation at 35°C for 10 min, the reaction was terminated by addition of 125 μl Norit A (Serva) suspension 4% in 0.1 M HCl. After shaking for 1 min and standing in ice for 10 min the charchoal was removed by centrifugation at 4000 × *g*. For counting, 0.1-ml samples of the supernatant containing the $^{32}P_i$ were used. Parallel assays without DNA were performed. 1 unit represents that amount of enzyme which degrades 1 nmol of ATP to ADP and P_i in 10 min, under the conditions mentioned above.

ATP-dependent DNAase assay. The reaction mixture contained in 75 μl, 10 nmol nucleotide-equivalent [^{3}H]DNA, 15 nmol ATP, 3 μmol $MgCl_2$, 3.75 nmol Tris-HCl buffer (pH 8.0) and enzyme. A control assay without ATP was run simultaneously. Incubations were for 20 min at 37°C, stopped by addition of 100 μl 2 M perchloric acid and 25 μl of 5 mg/ml bovine serum albumin. After centrifugation at 5000 × *g* for 10 min aliquots of 100 μl were taken for radioactivity measurements.

Enzyme abbreviations. The convention of using the genus name and the first two letters of the species [19], and serial numbering with Roman numerals of *E. coli* exonucleases [20], served as a model in our abbreviations. For example, we denoted DNA-dependent ATPase I of *E. coli* as *E. coli* ATPase I and DNA-dependent ATPase I from *B. cereus* as *B. cereus* ATPase I. Conventionally accepted names such as helicases, rep protein and others, are also used.

Results

Purification of DNA-dependent ATPase II

B. cereus NRRL B-569 was grown in 5 l Casamine medium. The logphase cells ($6 \cdot 10^8$ cells per ml) were harvested by centrifugation and washed once with 0.5% NaCl/0.5% KCl solution and once with cold buffer A. Cells were resuspended by adding 2 ml of buffer A per g cells (wet weight). Cell suspensions were stored at −20°C. All other operations were carried out at 0—4°C and centrifugations were performed at 12 000 × *g* for 20 min unless otherwise stated. A summary of the purification procedure is shown in Table I. The total

TABLE I

PURIFICATION OF ATPase II

Fraction		Total protein (mg)	Total activity (kU)	Specific activity (kU/mg)
I	Extract	2620	—	—
II	40% $(NH_4)_2SO_4$ supernatant	2015	—	—
III	50% $(NH_4)_2SO_4$ precipitate	375	—	—
IV	1st DEAE-cellulose	56	24	0.4
V	2nd DEAE-cellulose	6.2	8.2	1.3
VI	ATP-Sepharose	0.3	5.2	17
VII	Concentration on DEAE-cellulose	0.04	3.4	85

recovery of ATPase II cannot be given prior to fraction IV as only a part of DNA-dependent ATPase activity is due to ATPase II [11].

The frozen cell suspension was slowly thawed at 4°C. 5-ml aliquots were disrupted with 10 pulses from a sonic 300 dismembrator (Artek). The debris was removed by centrifugation. The supernatant was centrifuged at 110 000 × *g* for 2 h. The supernatant was dialyzed against 200 vols. of buffer A (fraction I).

Freshly prepared 10% streptomycin sulphate solution in buffer A was slowly added by constant stirring over a 20 min period to fraction I. The volume of the streptomycin sulphate was 0.20 that of fraction I. After mixing for another 20 min the suspension was centrifuged and the pellet containing the nucleic acids was discarded. Solid pulverized $(NH_4)_2SO_4$ was added to the supernatant over a 20 min period to reach 40% saturation. After stirring for another 20 min the suspension was centrifuged and the supernatant was saved (fraction II).

Solid $(NH_4)_2SO_4$ was added to fraction II to 50% saturation. After centrifugation, as described above the precipitate was dissolved in buffer B and dialyzed against the same buffer until the conductivity of the enzyme solution reached that of buffer B (fraction III). Fraction III was applied to a DEAE-cellulose colum (2.5 cm^2 × 14 cm) equilibrated with buffer B. After washing with 60 ml of buffer B the proteins were eluted with a linear salt gradient of 0—0.45 M NaCl in buffer B (300 ml total volume). As shown in Fig. 1, three different DNA-dependent ATPases were resolved by this chromatography. The activities eluting at 0.18, 0.24 and 0.4 M NaCl concentrations were denoted as ATPase I, II and III, respectively. ATPase I represents a previously described DNA-dependent ATPase activity [11]. Active fractions of ATPase II (61—79) were pooled and dialyzed against buffer B (fraction IV).

Fraction IV was loaded onto a second DEAE-cellulose column (2.5 cm^2 × 4 cm) washed with 30 ml of 0.05 M NaCl in buffer B and eluted with a linear

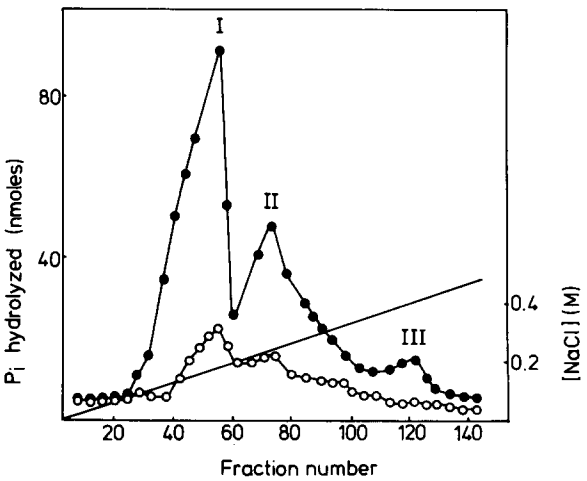


Fig. 1. DEAE-cellulose chromatography of DNA-dependent ATPases from *B. cereus*. ATPase activities were measured in the absence (○———○) and in the presence (●———●) of T7 DNA. ———, NaCl concentration gradient. Chromatography and assay were carried out as described in the text and in Materials and Methods.

gradient (200 ml total volume) from 0.05 to 0.35 M NaCl. Residual ATPase I eluted at 0.17 M NaCl. ATPase II eluted at 0.23 salt concentration, and was free of ATPase I, but contained some single-strand specific DNAase and DNA-independent ATPase activities as well. Fractions containing ATPase II activity were combined, and dialyzed against buffer B (fraction V).

In order to remove the contamination that went together with ATPase II in all the previous steps, fraction V was subjected to ATP-Sepharose affinity chromatography. The column (2 $cm^2 \times 1$ cm) was equilibrated with buffer B. Bound ATPase II was eluted by a linear gradient (40 ml total volume) of 0—0.6 M NaCl. ATPase II activity eluted as a single peak at 0.4 M NaCl concentration, while DNA independent-ATPase activity appeared earlier, at 0.25 M salt concentration. It was found that ATPase II did not contain detectable DNAase activity after this step of purification. Active fractions of ATPase II were pooled and dialyzed against buffer B (fraction VI).

Fraction VI was loaded onto a DEAE-cellulose column (0.8 $cm^2 \times 1$ cm) equilibrated with buffer B. The column was batch-washed with 2 ml buffer B and with 2 ml of the same buffer containing 0.1 M, 0.2 M, 0.3 M and 0.4 M NaCl, respectively. ATPase II activity eluted in the 0.3 M fraction. This fraction was dialyzed against buffer B (fraction VII). Subsequent experiments were carried out with fraction VII.

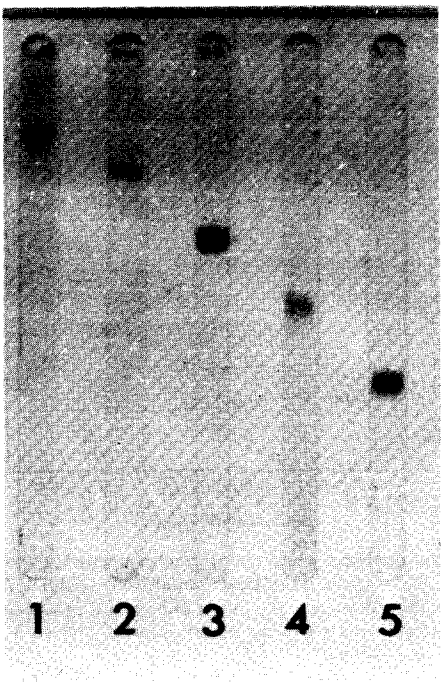
Properties of the purified enzyme

Molecular weight determination. Sodium dodecyl sulphate (SDS) acrylamide gel electrophoresis of fraction VII showed a sharp protein band (Fig. 2). Relative to standards on SDS-acrylamide rod gels ATPase II migrated as a single polypeptide of molecular weight 84 000. Since a faint band was visible below the single sharp band (Fig. 2, frame 1) the homogeneity of fraction VII was quantitatively estimated with a Joyce-Loebl densitometer after SDS-acrylamide gel electrophoresis. The surface of the enlarged densitometric profile was measured using a MOM planimeter. The single *B. cereus* ATPase II peak represented 94% of the total surface, suggesting that the enzyme had been purified over 90% and that the faint band represented not more than 6% of the total.

Assay conditions. Optimal conditions for the ATPase reaction were tested. It was found that ATPase II had nearly identical activities in the range of pH 7.5 and 9.5. The enzyme required a bivalent cation for its catalytic function. ATPase II was stimulated by $MgCl_2$ and $CaCl_2$ with an optimum at 1 mM, $MnCl_2$, $CuCl_2$ and $CoCl_2$ were less effective.

Requirement of ATP. ATPase II activity was measured at different concentrations of ATP at two enzyme concentrations in order to determine the V and the apparent K_m values for hydrolysis of ATP. The enzyme became saturated at about 1 mM ATP, and the value of the K_m estimated from the Lineweaver-Burk plot was 0.2 mM.

The ATP analogues, adenosine 5′-(β,γ-imido)-diphosphate and adenosine 5′-(β,γ-methylene)-diphosphate were tested for their inhibitory effect on ATP phosphohydrolysis. The imido analogue competed with ATP for the enzyme and caused a 74% inhibition at 2.5 mM concentration, whilst the methylene analogue of ATP caused only 17% inhibition at the same concentration. As



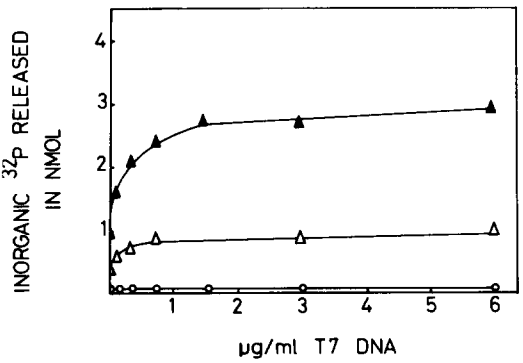


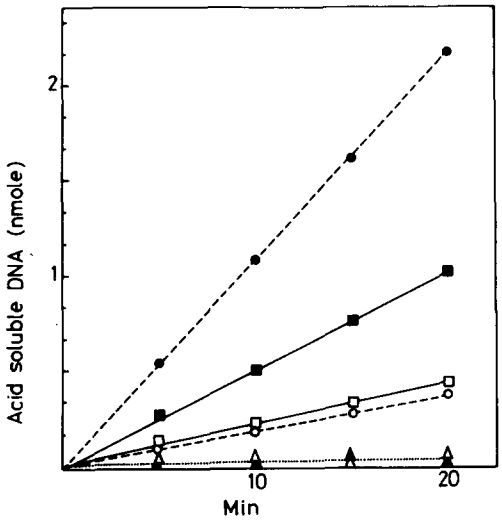
Fig. 2. Sodium-dodecyl sulphate-acrylamide gel electrophoresis of *B. cereus* ATPase II. Electrophoresis was carried out according to Weber and Osborn [14], fraction VII and protein standards were treated with SDS, electrophoresis in 10% polyacrylamide gel was for 8 h at 2 mA/gel at 20°C. Fraction VII (3 µg) (1), bovine serum albumin (2), glyceraldehyde phosphate dehydrogenase (3), trypsin (4) and ribonuclease (5) were run separately. The dye front was broomophenol blue. Gels were stained with Coomassie blue.

Fig. 3. ATPase II activity as a function of DNA cofactor concentration. ATPase II was assayed as described in Materials and Methods. T7 native DNA (△———△) and T7 denatured DNA (▲———▲) were used for activation. ATPase activity in the presence of T7 native DNA treated with S_1 nuclease (○———○).

potential inhibitors of DNA synthesis nalidixic acid, rifampicin, actinomycin D and ethidium bromide were used. Nalidixic acid and rifampicin had no inhibitory effect, whereas actinomycin D had a slight and ethidium bromide a strong inhibitory effect on the splitting of ATP by ATPase II.

DNA cofactor requirement. Single-stranded DNA is the preferred cofactor for the enzyme, however, there is considerable ATPase activity in the presence of linear double-stranded DNA as well. ATPase activity could be enhanced by double-stranded DNA treated with DNAase I. RF I of PM 2 DNA and RNA are not cofactors of ATPase II. DNA-dependent ATPase II as a function of single- and double-stranded DNA shows a regular saturation curve (Fig. 3). The half-saturation for denaturated T7 DNA was 0.12 µg/ml.

DNA unwinding. Strand separation catalysed by DNA helicases and rep protein is usually measured by following the generation, in the presence of ATP, of single-stranded DNA from duplex substrates in an S_1 nuclease digestion assay [10,21]. Since, in our hands, S_1 nuclease was inhibited by ATP, a necessary component of the assay mixture, we used micrococcal nuclease instead of S_1 nuclease. Fig. 4 demonstrates that ATPase II in itself had no detectable DNAase activity either towards native or heat-denatured DNA. The basal



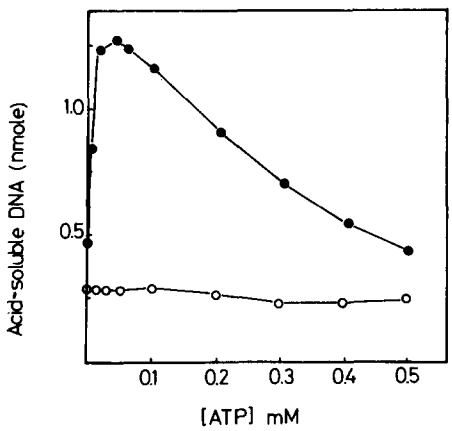


Fig. 4. The effect of ATPase II on the activity on micrococcal nuclease. Micrococcal-nuclease activity was detected under standard conditions both with native (○- - - - -○) and heat denatured [^{3}H]DNA (●- - - - -●) as substrate. DNAase-activity of ATPase II in the absence (△· · · · ·△) and in the presence (▲· · · · ·▲) of 30 nmol of ATP. Micrococcal-nuclease activity in the presence of 40 units of ATPase II (□———□), and 40 units of ATPase II plus 30 nmol of ATP (■———■).

Fig. 5. Effect of ATP concentration on micrococcal-nuclease activity stimulated with ATPase II. Standard micrococcal nuclease assay was carried out by using indicated ATP concentrations in the absence (○———○) and in the presence of 40 units of ATPase II (●———●).

DNAase-activity of micrococcal nuclease was stimulated about 3-fold in the presence of ATPase II and ATP. Control reaction with denatured DNA did not cause stimulation of the ATP-dependent DNAase activity of ATPase II. Optimal concentration for the unwinding of DNA was 50 μM (Fig. 5). Unexpectedly, no unwinding occurred at concentrations higher than 0.5 mM ATP, in spite of the fact that the hydrolysis of ATP reaches its saturation level at only about 1 mM ATP concentration.

Discussion

This paper reports the separation of three DNA-dependent ATPases, denoted *B. cereus* ATPase I, II and III, from extracts of *B. cereus* cells. ATPase I, described earlier [11], is the major enzyme among these ATPases, representing 60% of the total activity. ATPase II represents 35% and ATPase III is a minor component (5%). *B. cereus* ATPase II has been purified to homogeneity. The enzyme has a specific activity of $2 \cdot 10^6$ units per mg protein and has a molecular activity of 16 800 hydrolysed ATP molecules by a single molecule of enzyme per min. The K_m for ATP is 0.2 mM.

Adenosine 5′-(β,γ-imido)-diphosphate is an effective inhibitor of *B. cereus* ATPase II. Adenosine 5′-(β,γ-methylene)-diphosphate, another analogue of ATP, is ineffective as an inhibitor of this enzyme, similarly to helicase I and II of *E. coli* [9,22]. Actinomycin D and ethidium bromide, forming a stable com-

plex with DNA, interfere with its normal function as cofactor in the ATPase reaction.

Based upon various criteria, *B. cereus* ATPase II differs from *E. coli* ATPases. The molecular weight of *B. cereus* ATPase II (84 000) is similar to that of *E. coli* ATPase II (86 000) [6] but distinct from those of helicase I [23], helicase II [9] and rep protein [4]. In spite of several similarities between *E. coli* ATPase II and *B. cereus* ATPase II, there is an essential difference between them. *E. coli* ATPase II requires double-stranded polynucleotide as cofactor, whereas *B. cereus* ATPase II is more specific for single-stranded DNA. In contrast to rep protein, pancreatic DNAase I-treated DNA is a suitable cofactor for *B. cereus* ATPase II. *B. cereus* ATPase II is probably not a gyrase since nalidixic acid, an inhibitor of gyrase, has no effect on it. *B. cereus* ATPase II is also distinct from ATP-dependent DNAase as it has no DNAase activity.

Because of the inhibitory effect of ATP on S_1 nuclease activity, this enzyme was replaced by micrococcal nuclease in the unwinding assay. Partially melted regions of DNA are cleaved at 'dA-T' rich regions by micrococcal nuclease [24, 25], so it can be used as a conformational sensitive probe of *B. cereus* ATPase II.

Strand separation of DNA by means of *B. cereus* ATPase II was observed in a relatively narrow range of ATP concentration. At ATP concentrations higher than 0.5 mM the unwinding of duplex was inhibited. This probably means that a change in ATP concentration influences strand separation to ensure a regulated melting which may be essential for strand separation of DNA.

In the course of these studies a contaminating DNAase was separated. This DNAase co-purified with *B. cereus* ATPase II during several steps of purification. Preliminary experiments with *B. cereus* ATPase II and the separated DNAase specific for single-stranded DNA, point to the ATP-dependent co-operation of these enzymes. The co-operation seems to be similar to the unwinding reaction and appears as an ATP-dependent DNAase activity. It points to the possibility that ATP-dependent DNAase consists of these two enzymes closely associated with DNA.

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