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A DNA-DEPENDENT ATPase FROM BACILLUS SUBTILIS

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SUMMARY: A DNA-dependent ATPase (molecular weight 68000) has been purified from extracts of B. subtilis. The enzyme shows specificity for single-stranded DNA and for hydrolysis of ATP (Km 0.4 mM). Similarities with the rep gene product from E.coli are discussed.

A requirement for ATP has been shown for replication of DNA in various in vitro systems derived from E. coli (1-3). Recently several groups have purified probably identical single-stranded DNA-dependent ATPases from E. coli (4-6) and the involvement of this enzyme (the rep protein) in replication of ØX174 DNA has been demonstrated (5). ATP has also been shown to be required for DNA replication in toluenised cells of B. subtilis (7) and it was of interest to investigate whether a similar enzyme was present in this bacterium. Addition of single-stranded DNA to a crude extract from B. subtilis resulted in a 100% stimulation of the background ATPase activity. The DNA-dependent ATPase was purified and shown to resemble the E. coli enzyme in several respects.

MATERIALS AND METHODS

Buffer A: trisCl pH 8.0 (50 mM), 2-mercaptoethanol (10 mM), EDTA (1 mM), KCl (0.2 M). Buffer B: trisCl pH 8.0 (50 mM). Buffer C: trisCl pH 8.0 (50 mM), KCl (0.2 M). Buffer D: trisCl pH 8.0 (50 mM), glycerol (30% v/v), $MgCl_2$ (1 mM).

ATPase activity was assayed by measuring release of ^{32}Pi from [γ-^{32}P]ATP using a method based on that of Rosamond and Lunt (8). The reaction mixture (0.1 ml) contained trisCl pH 7.5 (20 mM), $MgCl_2$ (0.5 mM), heat denatured E. coli DNA (200 µM), [γ-^{32}P]ATP (1 mM) and enzyme. After 15 min incubation at 37°C the reaction was terminated by addition of ice-cold HCl, charcoal and bovine

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serum albumin (0.1 ml each) and the radioactivity which did not adsorb to charcoal was determined as in (8). ATPase activity in the absence of DNA was subtracted from activities measured in the early stages of purification. No DNA-independent activity was found in fractions 4 and 5. One unit of enzyme is defined as the amount that catalyses the hydrolysis of one nmole ATP in 15 min under these conditions. The assay was found to be linear with respect to time and enzyme concentration (up to 15 units present in assay).

For non-radioactive nucleoside triphosphate hydrolysis, P_i release was measured by a modification of the Chen method (9).

Nuclease assays were carried out according to the method of Rosamond and Lunt (8). The reaction mixture (0.1 ml) contained trisCl pH 7.5 (20 mM), $MgCl_2$ (0.5 mM), ATP (1 mm), [^{3}H]labelled DNA from E. coli (native or heat denatured) (200 μM) and enzyme.

DNA was prepared from E. coli K12.1100 as described in (8). Denaturation was carried out by heating at 100°C for 15 min followed by rapid cooling in ice. DNA concentrations are expressed in terms of nucleotide equivalents.

DNA was linked to Sepharose by the method of Arndt-Jovin et al (10). CNBr-Sepharose 4B (300 mg) was shaken overnight at room temperature in 50 ml of 10 mM potassium phosphate buffer pH 8 containing 3 mg/ml of heat denatured Herring Sperm DNA (Sigma). The final concentration of DNA retained by the column was 10-15 mg/ml.

Protein concentrations were determined by the method of Lowry (11) or by UV absorption.

Purification:

A 1 l overnight culture of B. subtilis 168 in Penassay broth was added to 18 l of fresh medium to a cell density of 0.04 mg dry wt/ml. Cells were grown with forced aeration to 0.45 mg dry wt/ml and harvested in a Sharples continuous-flow centrifuge. Cell paste (33 g wet wt) was resuspended in buffer A (200 ml) and sonically disrupted in a Mullard L560 sonicator at 2.5 A output for a total of 6 min. The resulting extract was centrifuged at 30000 g for 40 min and the supernatant fluid centrifuged again at 73000 g for 4 hr. The final supernatant fluid was retained (fraction 1).

Solid $(NH_4)_2SO_4$ was added slowly to fraction 1 to give a 30% saturated solution. Stirring was continued for 20 min and the resulting precipitate was removed by centrifuging at 10000 g for 30 min. Further $(NH_4)_2SO_4$ was added to the supernatant to give a 50% saturated solution. After 20 min stirring, the precipitate was collected by centrifuging as before and resuspended in 25 ml of buffer B (fraction 2). Fraction 2 was dialysed overnight against buffer B and then applied to a column of DEAE-cellulose (3.5 cm x 9 cm^2) equilibrated with buffer B. The column was washed with buffer B and bound protein was eluted with a 300 ml linear gradient of 0-0.4 M KCl in buffer B. A single peak of DNA-dependent ATPase activity was eluted at 0.15-0.2 M KCl - well separated from nucleic acid which was eluted around 0.35 M

KCl. Fractions which contained activity were combined (fraction 3).

$(NH_4)_2SO_4$ was added to fraction 3 to give a 60% saturated solution and the resulting precipitate was resuspended in 3 ml of buffer C. A column of Sephadex G-100 (63 cm x 4.9 cm^2) was equilibrated with buffer C. Concentrated fraction 3 was layered onto the top of the column which was then washed with 400 ml of buffer C at a flow rate of 20 ml/hr. Fractions which contained activity were pooled and dialysed against buffer D (fraction 4).

A column of Sepharose-linked single-stranded DNA (1 cm x 1 cm^2) was equilibrated with buffer D and fraction 4 was applied to the column. After washing with buffer D less than 10% of the protein remained on the column - this fraction contained 80% of the DNA-dependent ATPase activity. A linear gradient of 0 - 0.5 M KCl in buffer D was applied to the column and the enzyme was eluted in a broad peak between 0.2 and 0.3 M KCl. Fractions which contained activity were pooled (fraction 5).

All subsequent experiments were conducted with fraction 5 which had a half-life of one month when stored at -20°C. Protein yield was insufficient for electrophoretic analysis.

RESULTS

The Sephadex column employed in the purification procedure was calibrated using proteins of known molecular weight (bovine serum albumin, ovalbumin, trypsin, RNase A)(12). The elution volume of the DNA-dependent ATPase corresponded to that of a protein with molecular weight 68000.

No ATPase activity was detectable in the absence of DNA. Maximum activity was obtained with heat denatured DNA although some activity was found in the presence of native DNA (Fig. 1). RNA did not stimulate ATPase activity. No nuclease activity was found with native or denatured DNA in the presence or absence of ATP.

The effect of variation in ATP concentration is shown in Fig. 2a. The Km obtained from the Lineweaver-Burk plot (Fig. 2b) was 0.4 mM. The enzyme showed specificity for ATP although a small amount of activity was found with other nucleoside triphosphates (Table 2).

The enzyme was stimulated by addition of $MgCl_2$ with an

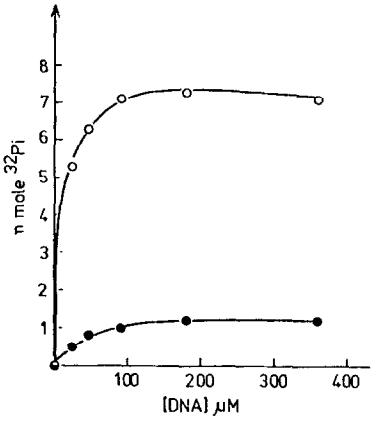

Fig. 1 Stimulation of ATPase activity by native (●) and heat denatured (O) DNA.

TABLE 1. Purification Procedure

Fraction	Step	Vol (ml)	Total protein (mg)	Total activity (10^3 units)	Specific activity (10^3 units/mg)
1	Crude extract	150	1800	250	0.14
2	30-50% $(NH_4)_2SO_4$ fraction	25	225	1450	5.5
3	DEAE-cellulose	18	47	560	12
4	Sephadex G-100	18	4	210	53
5	DNA-Sepharose	15	0.03	15	500

optimum at 0.5 mM - inhibition occurred at Mg^{2+} concentrations above 1 mM. EDTA (1 mM) inhibited the enzyme. Addition of $MnCl_2$ and $CoCl_2$ at 0.5 mM stimulated the enzyme to 70% and 60% respectively of the optimum level. $CaCl_2$ did not stimulate the enzyme.

The optimum pH for the enzyme was found to be 7-7.5

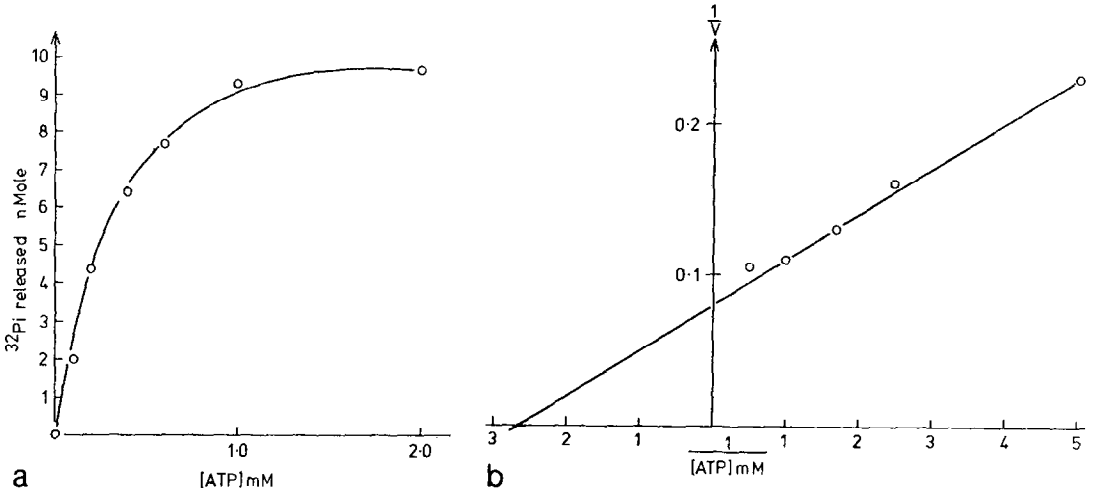

Fig. 2a Variations of ATPase activity with ATP concentration.
2b Lineweaver-Burk plot of data from Fig. 2a.

TABLE 2. Nucleoside Triphosphate Specificity

Nucleoside triphosphate	Activity
ATP	1.0
GTP	0.2
UTP	0.0
CTP	0.1

All nucleoside triphosphates were present at a concentration of 1 mM in the standard assay system. Activities are expressed relative to ATPase.

DISCUSSION

The enzyme described in this paper resembles the *E. coli rep* protein in a number of characteristics:

(1) Specificity for single-stranded DNA.

(2) Lack of nuclease activity.

(3) Specificity for ATP. With the *rep* protein some workers

have found this to be strict (6) while others (4) have found some activity with other nucleoside triphosphates as is the case with the B. subtilis enzyme. The Km for ATP is higher for the B. subtilis enzyme (0.4 mM) than for the E. coli enzyme (0.15 mM) (6).

(4) Molecular weight. The molecular weight (68000) is similar to that reported for the E. coli enzyme (69000 (4), 68000 (5), 75000 (6)).

The similarity between this enzyme and the E. coli rep protein suggests that it may be involved in DNA replication. ATP-dependent unwinding of duplex DNA involving the rep protein has been demonstrated in the ØX174 system (5) and also using partial duplex molecules (13). This type of enzyme may be required to utilise the energy of ATP hydrolysis to unwind the DNA duplex (14). It will be interesting to investigate the activity of this enzyme in DNA replication mutants of B. subtilis and at various stages of the growth cycle.

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