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MEMBRANE ATPase OF *BACILLUS SUBTILIS*

I. PURIFICATION AND PROPERTIES

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Summary

The membrane ATPase (EC 3.6.1.3) of *Bacillus subtilis* can be solubilized by a shock-wash process. Two procedures for purifying the solubilized enzyme are reported. A protease inhibitor, phenylmethane sulfonylfluoride, was introduced in the solubilization and purification step.

The resultant ATPase purified by density gradient centrifugation has a molecular weight of 315 000, an $s_{20,w}$ of 13,4 and an ámino acid composition very similar to bacterial ATPases already studied.

After exposure to polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (SDS), or 8 M urea or SDS-urea, the purified ATPase can be dissociated in two non-identical subunits of molecular weights 59 000 (α) and 57 000 (β) with different charges.

Kinetic studies showed that Ca^{2+} or Zn^{2+} are required for ATPase activity, although Mg^{2+} was uneffective. At optimal Ca^{2+} concentration, the Mg^{2+} has an inhibitory effect. The K_m for ATP is 1.3 mM. Inhibitors of the oxydative phosphorylation, of the mitochondrial ATPase and of the $(Na^+ + K^+)$-ATPase are studied.

Introduction

ATPases have been detected in a variety of membranes and envelope preparations from Gram-positive and Gram-negative bacteria. The ATPases of several bacteria have been obtained in soluble form and they appear to be relatively homogeneous. Bacterial, mitochondrial and chloroplast membrane ATPases are similar in many of their properties and in their structure. It is clear that information on the properties of the purified soluble enzyme will be needed to understand the functions of the ATPase. In the literature covering bacterial

Abbreviation: SDS, sodium dodecyl sulphate.

ATPases *, there is a general agreement that there are two major types of subunits, α and β, but there is lack of agreement with regard to the presence of minor subunits. It is a prevailing belief that the reason for the variance in the minor subunit composition among the different ATPases is that they are loosely associated with the major subunits and have a tendency to be lost during isolation and purification. Another hypothesis is valid: proteolysis by membrane proteases of the major subunits or of membrane proteins can take place during different stages of the enzyme preparation. In the study of the strict aerobe *Bacillus subtilis*, the main difficulty was the presence of exoproteases such as subtilisin and the use of a protease inhibitor was absolutely necessary. This work reports the molecular and some biochemical properties of the soluble ATPase from protoplast membrane of *B. subtilis*.

Materials and Methods

Microorganism and preparation of cytoplasmic membrane

B. subtilis (*leu*$^-$ *meth*$^-$ *threo*$^-$) was grown in an enriched medium at 37°C in a New Brunswick Fermentor Drive Assembly (220 rev./min; pressure, 2 bars). The cells were harvested in the log phase by centrifugation at 14 000 × *g* for 15 min.

The Rosenthal and Matheson method [1] was used with some modifications for the preparation of cytoplasmic membranes: the cells were washed twice in a 50 mM Tris · HCl pH 7.5, buffer, containing 10 mM $MgCl_2$. They were then stabilized in a hypertonic medium containing 200 mM sucrose/ 10 mM $MgCl_2$/0.1 mM phenylmethane sulfonyl fluoride in a 50 mM Tris · HCl, pH 7.5, buffer. Lysozyme was added to a final concentration of 500 µg/ml, converting the bacteria into protoplasts after 45 min at 37°C.

This conversion was controlled by examination under a phase contrast microscope. The protoplasts were harvested by centrifugation at 18 000 × *g* for 20 min. The resulting pellet was suspended in a 50 mM Tris · HCl, pH 7.5, buffer containing 10 mM $MgCl_2$ and 0.1 mM phenylmethane sulfonyl fluoride. Crystallized DNAase was then added to a final concentration of 40 µg/ml. After 30 min incubation at 37°C, the membranes were separated from the soluble cytoplasmic fraction by centrifugation at 40 000 × *g*.

Release of ATPase from cytoplasmic membranes

A modification of the method of Munoz et al. [2] was used as applied by Mirsky and Barlow [3] for *Bacillus megaterium*. The membrane pellet was washed 3 times in a 50 mM Tris · HCl, pH 7.5, buffer containing 0.1 mM phenylmethane sulfonyl fluoride. Respective centrifugations were carried out at 40 000 × *g* for 30 min. The last pellet was suspended in a 5 mM Tris · HCl, pH 7.5, buffer containing 0.1 mM phenylmethane sulfonyl fluoride, thoroughly homogenized and then centrifuged for 30 min at 40 000 × *g*. The resulting pellet underwent the same procedure. The two final supernatants, S_6 and S_7, contained most of the ATPase. These were pooled, RNAase added to a final concentration of 1 µg/ml and the mixture centrifuged at 120 000 × g for 2 h. The resulting supernatant constitutes the crude soluble ATPase.

* For general studies of bacterial ATPases see refs. 25 and 35.

Assay of ATPase activity

ATPase activity was measured by the liberation of P_i from ATP in a reaction mixture containing, in a final volume of 2 ml, 4 μmol ATP/100 μmol Tris · HCl, pH 7.5, buffer/4 μmol $CaCl_2$/50 μl soluble ATPase (about 50—30 μg protein for a crude soluble ATPase). In experiments where activation by Zn^{2+} was measured, 2 μmol of $ZnCl_2$ were substituted for $CaCl_2$ in the assay mixture. The amount of P_i released was measured according to the procedure of Baginski et al. [4] under conditions previously reported by Monteil et al. [5].

Proteins were determined by the procedure of Lowry [6], using bovine serum albumin as a standard and by the procedure of Ehresmann [7], based on measurements of absorbance at 228.5 and 234.5 nm.

One unit of enzyme activity is defined as that amount of protein which liberates 1 μmol P_i per min. Specific activity is expressed as units/mg protein.

ATPase purification

Two different procedures for the purification of the crude soluble ATPase were assayed.

1. Filtration on acrylamide-agarose (Ultrogel) [8]. Ultrogel AcA 2-2 and Ultrogel AcA 3-4 separated proteins of molecular weight between 60 000 and 1 000 000 and between 40 000 and 440 000, respectively. Chromatography was carried out at room temperature. Colums (2 × 40 cm) were equilibrated with 50 mM Tris · HCl, pH 7.5, buffer containing 0.1 mM phenylmethane sulfonyl fluoride and 5% glycerol and eluted with the same buffer. 2-ml fractions were collected at a flow rate of 10 ml/h. The effluent was monitored continuously at 280 nm for proteins. Aliquot fractions of 100 μl were tested for ATPase activity.

2. Linear glycerol gradients. A modification of the procedure of Mirsky and Barlow [9] was used. 1.5-ml aliquot fractions of crude soluble ATPase were layered over six 40 ml linear glycerol gradients (7—30%, w/v) containing 50 mM Tris · HCl, pH, 7.5, and 0.1 mM phenylmethane sulfonide fluoride. The gradient tubes were then centrifuged at 25 000 rev./min for 24 h at 14°C, using an SW 27 rotor. Fractions of 1.5 ml were collected from the top of the tube with an automatic density gradient fractionator at a flow rate of 1.5 ml/min. The effluent was monitored continuously at 280 nm for proteins. Aliquot fractions of 25 μl were tested for ATPase activity.

Analytical ultracentrifugation

Sedimentation coefficient was measured by ultracentrifugation analysis in a Spinco Model E analytical ultracentrifuge, equipped with an AN-E rotor, and a schlieren system. Centrifugations were run at 20.2°C in the single-sector cells (16 mm) with rotation speed of 47 660 rev./min, at ATPase concentrations between 2 and 3 mg/ml in 50 mM Tris · HCl, pH 7.5. The position of the sedimenting boundary was recorded by taking photographs at intervals of 8 min.

Amino acid analysis

Samples containing 0.5—1 mg/ml of enzyme were lyophilized and hydrolyzed with 6 M HCl at 105°C for 24, 36 and 48 h in sealed tubes, under

vacuum. Analysis of the hydrolysate was performed by ion exchange chromatography on a Beckmann Multichrom Liquid Column Chromatography 42-55. The techniques of Deveny [10] or of Benson and Patterson [11] were used. Cystein content was determined after performic acid oxydation of the enzyme using the method of Moore [12].

Polyacrylamide disc gel electrophoresis

Different electrophoretic procedures were used for this study: electrophoresis in non-dissociating conditions at alkaline pH, according to the general procedure described by Davis [13], was carried out on separating gels with 6.6% acrylamide and 0.18% *N,N'*-methylene bisacrylamide (w/v). The polymerisation was performed at 20° C for 1 h in 0.38 M Tris · HCl, pH 8.8. Electrophoreses were run at room temperature in 20 mM Tris/0.2 M glycine buffer (pH 8.5) for 4—5 h, at 2—4 mA per gel. Bromophenol Blue was used as tracking dye. Samples were layered onto the gels with or without prior treatment with 1% β-mercaptoethanol or 10 mM dithiothreitol. Proteins were stained with Coomassie Blue, and ATPase activity specifically located on the gel through an enzyme reaction previously reported by Monteil et al. [14].

Electrophoresis under dissociating conditions: (1) SDS-Tris-glycine system. According to the general procedure described by Maizel [15], electrophoresis was carried out on gels with acrylamide concentrations of 7.5 and 10% (acrylamide/*N,N'* methylene bisacrylamide = 37), and containing 0.1% sodium dodecyl sulphate. Samples were pretreated with 1% SDS + 1% β-mercaptoethanol or 10 mM dithiothreitol at 100° C for 2 min or at room temperature for 30 min. Electrophoresis was run in a 20 mM Tris/0.2 M glycine buffer, pH 8.5, containing 0.1% SDS. The current during stacking (upper gel) was 1 mA/gel and then increased to 2—3 mA/gel.

2. Urea-Tris-glycine system. Electrophoresis was carried out on gels of 7.5% acrylamide concentration containing 8 M urea. The gels and the reservoir buffer contained the same Tris/glycine buffer, pH 8.5 (20 mM Tris/0.2 M glycine). Samples were pretreated with 8 M urea and 1% β-mercaptoethanol.

3. SDS-urea-Tris-glycine system. The general procedure described by Swank and Munkres [16] was modified using gels with 7.5% acrylamide containing 0.1% SDS and 8 M urea. The polymerisation was performed in 20 mM Tris/0.2 M glycine buffer, pH 8.5. Samples were pretreated with 1% SDS, 8 M urea and 10 mM dithiothreitol. The electrode compartments contained Tris/glycine buffer, pH 8.5, and 0.1% SDS as in the gel. Electrophoresis was run at room temperature for 10 h at 0.25 mA/gel.

Results

ATPase solubilization. The results are shown in Table I, in terms of specific activity. The two supernatants S_6 and S_7, obtained by treating the membranes with 5 mM Tris · HCl, pH 7.5, contain the greatest amount of total enzyme and specific activity. Further washing with the same buffer yielded little ATPase.

We found that we could obtain more regular results by adding protease inhibitor to all stages of the solubilization process. Phenylmethane sulfonyl

TABLE I

SOLUBILIZATION OF ATPase FROM CYTOPLASMIC MEMBRANES OF *B. SUBTILIS*

For method, see text.

Fractions	Buffer	ATPase specific activity (units/mg protein)
Supernatants of washed membrane fractions		
S_3	50 mM Tris · HCl, pH 7.5/	0.017
S_4	0.1 mM phenylmethane sulfonyl fluoride	0.018
S_5		0.111
Washed membrane		
C_5	50 mM Tris · HCl, pH 7.5/ 0.1 mM phenylmethane sulfonyl fluoride	0.117
Supernatants containing the released ATPase		
S_6	5 mM Tris · HCl, pH 7.5/	4.970
S_7	0.1 mM phenylmethane sulfonyl fluoride	4.080
S_8		0.336
Depleted cytoplasmic membrane		
C_8	50 mM Tris · HCl, pH 7.5/ 0.1 mM phenylmethane sulfonyl fluoride	0.030
Supernatants $S_6 + S_7$ 120 000 × *g* (2 h)	Crude soluble ATPase	5.0 ± 10%

fluoride inhibits proteases with serine in the active centre such as subtilisin produced by *B. subtilis*.

The ATPase specific activity was enriched by centrifugation of the crude ATPase (S_6 and S_7) at 120 000 × *g* for 2 h. Monteil et al. [14] showed that this ultracentrifugation eliminated membrane particles and high molecular weight contaminants.

ATPase purification. Crude ATPase ($S_6 + S_7$) was concentrated to a protein concentration of 4—6 mg/ml and layered on the glycerol gradients or on an acrylamide-agarose column.

Figs. 1 and 2 display the proteins and ATPase activity profiles resulting from glycerol gradient centrifugation and acrylamide-agarose column (AcA 2-2) separation, respectively.

In Fig. 1, we note that the smaller protein peak, including fractions 13 to 19, corresponds exactly to enzyme activity, contrary to Fig. 2.

Compared to the ATPase fractions eluted from the acrylamide-agarose column, the ATPase fractions collected from the glycerol gradients have greater specific activity, implying greater purity. Glycerol gradients gave a better yield, with respect to the amount of protein layered on the gradient and the column. Possibly, a part of the enzyme is denaturated during the gel filtration. The glycerol gradient fractions 15 to 17 have optimal specific activity of 20—40 units/mg protein. ATPase of these fractions was used in tests where enzyme purity is an essential requirement.

Polyacrylamide gel electrophoresis enabled us to check the purity of the ATPase from these fractions. Whether or not the sample was pretreated with β-mercaptoethanol, the gels A and B (Fig. 3) yielded a single band with identi-

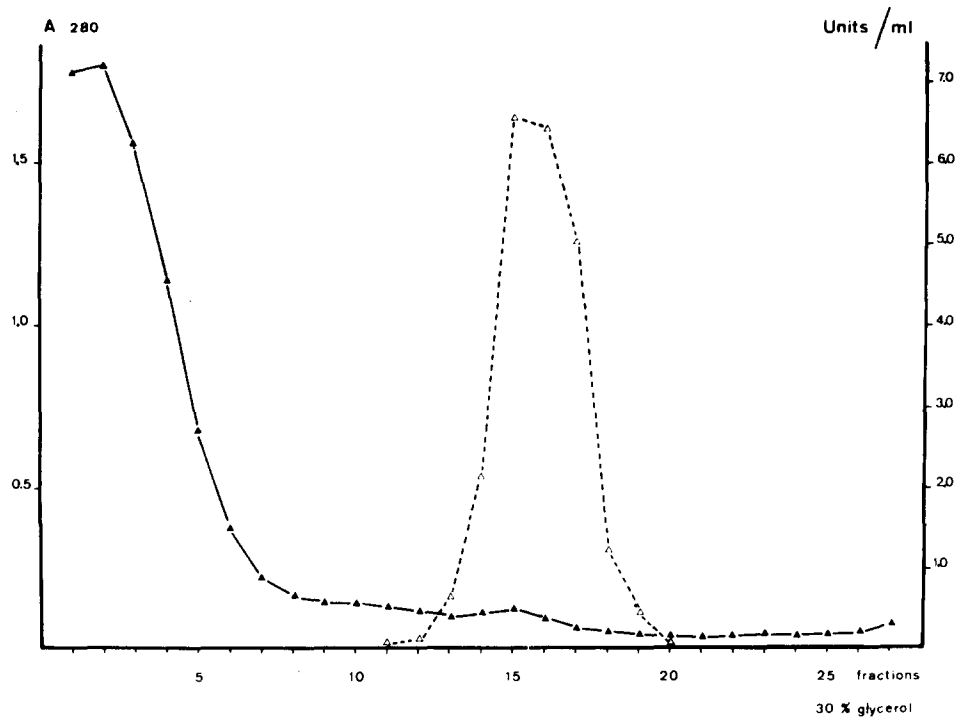

Fig. 1. Glycerol gradient centrifugation of ATPase. 1.5 ml of crude ATPase (4—6 mg/ml of protein concentration) was layered over a glycerol gradient and centrifuged, according to conditions described in Material and Methods. Fractions of 1.5 ml were collected and monitored continuously at 280 nm for proteins (▲———▲) and assayed for ATPase activity (△- - - - -△).

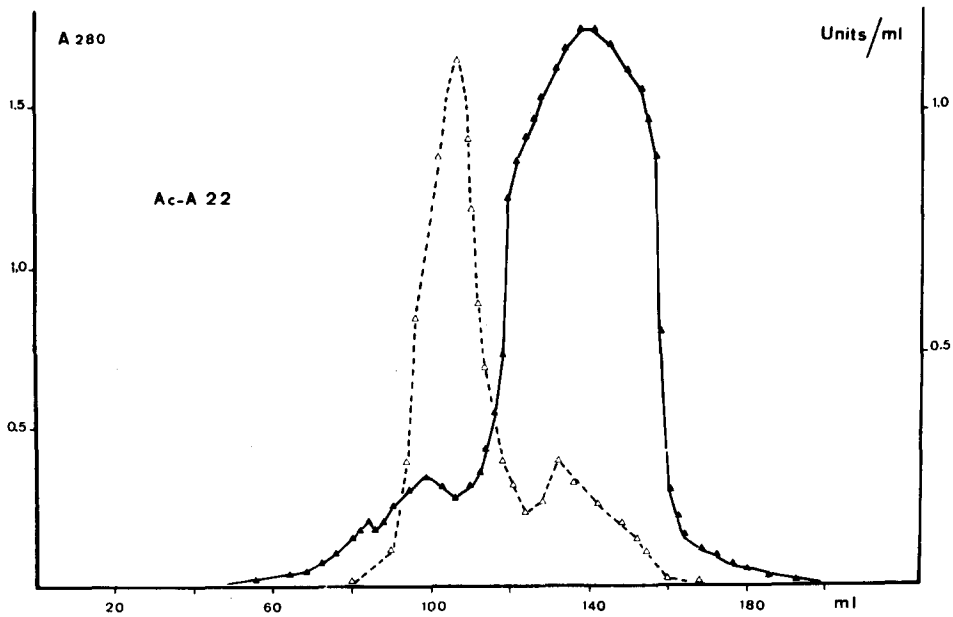

Fig. 2. Ultrogel AcA 2-2 filtration of ATPase. 3 ml of crude ATPase (4—6 mg/ml of protein concentration) was layered over a column of the type described in Material and Methods and eluted as indicated there. Fractions of 2 ml were collected. The effluent was monitored continuously at 280 nm for the proteins (▲———▲) and assayed for ATPase activity (△- - - - -△).

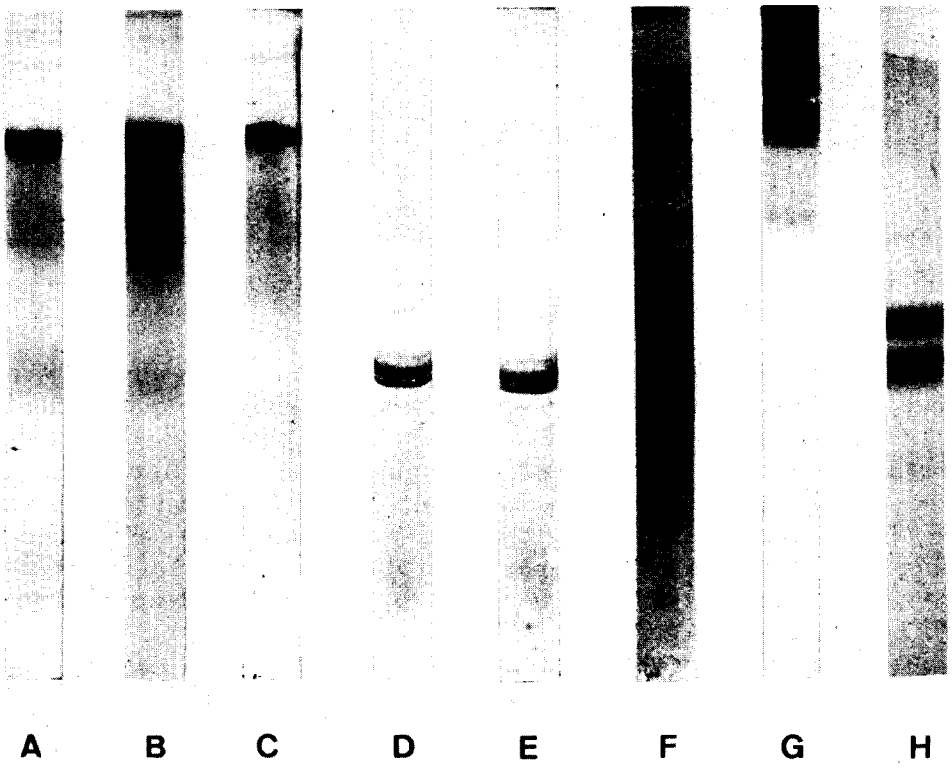

Fig. 3. Polyacrylamide gel electrophoresis of purified ATPase. Non-denaturating conditions: 20—30 μg protein were layered over the following gels A, B, and C (6.5% acrylamide). A, native ATPase; B, ATPase treated with 1% β-mercaptoethanol; C, ATPase stored for 8 days at 7°C in 20% glycerol. Denaturating conditions: effect of SDS: gel D (7.5% acrylamide) and gel F (10% acrylamide), ATPase (10 μg) treated by 1% SDS, 2 min at 100°C; gel E (7.5% acrylamide), ATPase (10 μg) treated by 1% SDS, 30 min at 20°C. Effect of SDS-urea: gel H (7.5% acrylamide), ATPase (50 μg) treated by 8 M urea, 1% SDS and 10 mM dithiothreitol. Effect of urea: gel G (7.5% acrylamide), ATPase (80 μg) treated with 8 M urea and 1% β-mercaptoethanol.

cal electrophoretic mobility. This band corresponded to ATPase identified on the gel by enzyme activity.

Analytical ultracentrifugation. Fig. 4 shows ATPase migration according to time. We found an $s_{20,w}$ sedimentation coefficient of 13.4 S (three runs).

Molecular weight. A sample containing ATPase, after partial purification on a glycerol gradient (Fig. 5) was applied to an acrylamide-agarose column (AcA 3-4). An approximate molecular weight of 315 000 was estimated for the native pure enzyme. Proteins with well-defined molecular weights were used as standards: ferritin, fibrinogen, catalase and aldolase.

Amino acid composition. The amino acid composition of *B. subtilis* ATPase expressed in mol/mol native enzyme and in mol% is reported in Table II. Cystein was determined as cysteic acid after performic acid oxidation. The results give a composition quite similar to that of bacterial ATPases [17—20].

Subunits and their molecular weight. From the results of polyacrylamide gel electrophoresis under denaturating conditions (see Fig. 3): SDS (gels D, E

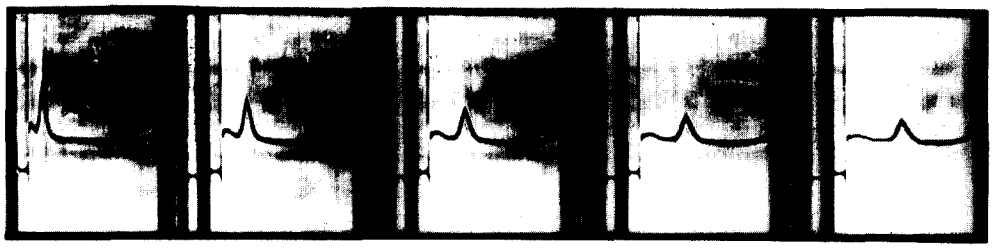

Fig. 4. Sedimentation velocity runs of purified ATPase by analytical ultracentrifugation according to conditions described in Material and Methods. The protein was sedimented from left to right.

and F), 8 M urea (gel G) and SDS-urea (gel H), we conclude that ATPase is made up of two subunits. The charges of these subunits are quite different, but their molecular weights are closely related. We occasionally observed an irregular array of very faint bands. Therefore, there is no proof that these bands are part of the native enzyme structure. We stress the fact that the number of these auxiliary bands was considerably decreased when phenylmethane sulfonyl fluoride was added up to all solubilization and purification steps and thus we consider them to be either contaminating proteins or residues of ATPase degradation.

Subunit separation was found to be more efficient in gel H (50 μg protein) where SDS (0.1%) was combined with 8 M urea, than in gels D and F (10 μg) where SDS is the sole denaturating factor. The acrylamide concentration was the same in all three gels (7.5%).

An evaluation of the molecular weight of these subunits was calculated by electrophoresis on polyacrylamide gel (7.5%) with SDS, comparing their

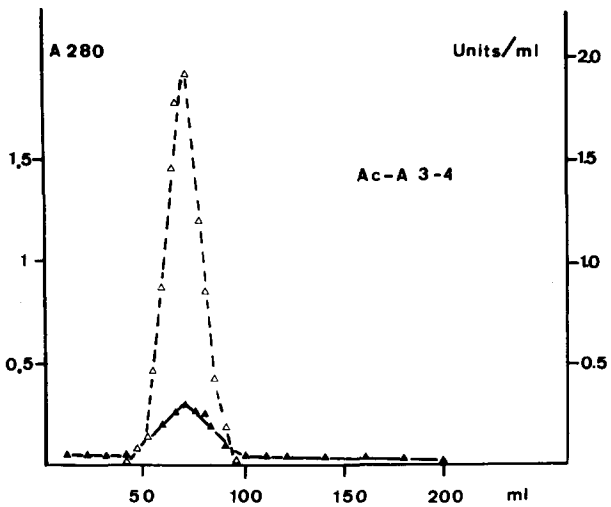

Fig. 5. Ultrogel AcA 3-4 filtration of ATPase after partial purification on a glycerol gradient. 2 ml of this ATPase (5 mg proteins/ml) were layered as previously for AcA 2-2. Fractions of 2 ml were also collected, assayed for ATPase activity (△- - - - -△) and monitored at 280 nm for the proteins (▲———▲). The volume of elution for ATPase is 62 ml.

TABLE II

AMINO-ACID COMPOSITION OF *B. SUBTILIS* ATPase

The conditions are described in the text. Cysteine was determined as cysteic acid after performic acid oxidation.

Amino-acid residue	*B. subtilis* ATPase	
	mol/mol native enzyme	mol/100 mol
Lysine	125	5.8 ± 0.2
Histidine	44	2.15 ± 0.2
Arginine	86	4.75 ± 0.7
Aspartic acid	258	10.15 ± 0.35
Threonine	154	5.85 ± 0.05
Serine	174	5.6 ± 0.2
Glutamic acid	223	11.04 ± 0.8
Proline	123	4.3 ± 0.2
Glycine	365	8.7 ± 0.5
Alanine	332	9.25 ± 0.15
Cysteine	3	0.1
Valine	244	8.35 ± 0.75
Methionine	25	1.17 ± 0.3
Isoleucine	154	6.00 ± 0.4
Leucine	228	9.5 ± 0.5
Tyrosine	42	2.4 ± 0.2
Phenylalanine	74	3.77 ± 0.4
$\frac{\text{Lys + Arg + His (basic)}}{\text{As + Glu (acidic)}}$		0.58
$\frac{\text{Polar}}{\text{Apolar}}$		1.5

electrophoretic mobilities with those of reference proteins with well defined molecular weights: bovine serum albumin, denatured catalase, and ovalbumin. The two molecular weights were 59 000 and 57 000, respectively. The ratio of the intensities of the two electrophoretic bands was 1 : 1. On the basis that the approximate molecular weight of *B. subtilis* ATPase is 315 000, we suggest the subunit formula $\alpha_3\beta_3$.

The role of divalent cations Ca^{2+}, Zn^{2+} and Mg^{2+}. In order to observe ATP hydrolysis by ATPase from *B. subtilis*, it is mandatory to add divalent cation to the medium. Ca^{2+} and Zn^{2+} present an equivalent optimal activation effect at pH 7.5. If we consider this activation to be 100%, Mg^{2+} activation based on the initial flow rate is only 30%. Mg^{2+} activates ATP hydrolysis during the first 2 min. After this time, the amount of released P_i does not seem to increase. The optimal Ca^{2+}/ATP and Zn^{2+}/ATP ratios are 1 and 0.5, respectively, with ATP saturating the enzyme. There is no optimal Mg^{2+}/ATP ratio. When 0.5—10 mM Mg^{2+} was added to Ca^{2+}, Mg^{2+} caused a 90% decrease in Ca^{2+}-activated ATPase activity.

Substrate specificity. We tested ATPase specificity for various nucleotides. Considering activity in the presence of ATP is 100%, trinucleotides of the pyridine type are hardly hydrolyzed at all (UTP and CTP $\simeq$ 6%). In the presence of GTP, the enzyme is about 30% active. We can therefore assume that the enzyme recognizes purine structure to a certain extent. ADP and AMP

TABLE III

STUDY OF SOLUBLE *B. SUBTILIS* ATPase INHIBITION BY VARIOUS COMPOUNDS

Compounds	Concentrations	Inhibition (%)
Dinitrophenol	1 mM	2
	2 mM	8
Carbonyl cyanide *P*-trifluoromethoxyphenylhydrazone	0.1 mM	22
	1 mM	100
Gramicidin	10 μg/ml	27.4
	20 μg/ml	60
Oligomycin	20 μg/ml	0
P-Chloromercuribenzoate	0.1 mM	10.6
	0.2 mM	13.6
N-Ethylmaleimide	2 mM	0
Iodoacetamide	1 mM	0
	2 mM	0
Ouabain	0.1 mM	0
	0.2 mM	0
N,N-Dicyclohexylcarbodiimide	0.1 mM	2
	0.2 mM	9
Sodium azide	0.5 mM	72.8
	1 mM	80.8
	2 mM	86.9
Concanavalin A	25 μg—100 μg/ml	0

are not hydrolyzed. The K_m for ATP is 1.3 mM, calculated with an optimal Ca^{2+}/ATP ratio of 1/1.

Inhibition of the soluble Ca^{2+} ATPase activity under various conditions. As shown in Table III, the effect of various compounds on the soluble Ca^{2+} ATPase was examined. 2.4-Dinitrophenol did not show any important effect of inhibition, but gramicidin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, also uncouplers of oxydative phosphorylation, by mitochondria, reduced the activity by 60 and 100%, respectively. Oligomycin and ouabain, which are inhibitors of mitochondrial ATPase and $(Na^+ + K^+)$-ATPase, respectively, had no effect on the Ca^{2+}-ATPase reaction.

An 80% inhibition of membrane-bound ATPase was obtained with *N,N*-dicyclohexylcarbodiimide, a specific inhibitor of ATPase at a concentration of approx. 10^{-6} M. However, 10^{-6}—10^{-4} M *N,N*-dicyclohexylcarbodiimide did not inhibit soluble ATPase activity. In contrast to membrane-bound ATPase of lymphocytes [21] and of *Escherichia coli* [22], concanavalin A did not show any effect on the soluble ATPase activity.

Soluble ATPase stability and enzyme conservation. When crude soluble ATPase is incubated at 18—20°C in a 5 mM Tris · HCl buffer, pH 7.5, containing 0.1 mM phenylmethane sulfonyl fluoride, it retains 80% of initial activity for 4 days.

After precipitation with 70—90% $(NH_4)_2SO_4$, the enzyme can be kept at 4°C for at least a month. At −20°C, the ATPase remains fully active for at least a month at a protein concentration of 2 mg/ml and in the presence of 50% glycerol and 2 mM of SH protector.

Enzymatic activity and electrophoretic behaviour (see Fig. 3, gel C) are

maintained for 7—15 days when purified soluble ATPase is incubated at 7°C in 20% glycerol.

Discussion

The principle of Abrams [23] involving selective release of ATPase from the membranes by washing them with low ionic strength Tris buffers, at neutral pH, in the absence of multivalent cations enabled us to solubilize ATPase from *B. subtilis*. The ionic strength of the solubilization buffers is similar to those used by Munoz and Salton for *Micrococcus lysodeikticus* [2,24] and by Mirsky and Barlow for *Bacillus megaterium* KM [3].

The ATPase-rich supernatants S_6 and S_7 have a specific activity which is comparable to supernatants S_5 and S_6 which contain the ATPase of *B. megaterium* KM solubilized by Mirsky and Barlow [3]. However, one must keep in mind the fact that the assays for determination of P_i are different in both cases. We applied two purification procedures to crude soluble ATPase and the ultracentrifugation in glycerol gradients gave the best results. The specific activity of purified ATPase compared with crude soluble ATPase is increased about five to ten times. This variability in degree of purification is a common problem with ATPases. We think that a possible explanation, already pointed out by Salton [25] may be unmasking effects resulting from membranes and/or inhibitors and regulatory proteins.

Purity was examined with the following criteria: absorbance at 280 nm, reflecting protein concentration; the correspondence between the protein peak and the peak of ATPase activity and electrophoresis on polyacrylamide gels. The $s_{20,w}$, for purified ATPase from *B. subtilis* is 13.4 S. It is interesting to note that *Streptococcus faecalis* ATPase has the same sedimentation coefficient [17]. Other bacterial ATPases have very similar $s_{20,w}$ values [18,9,26]. The molecular weight obtained by gel filtration was 315 000. This value is close to that found for other ATPases [14,17—19].

The study of the subunit structure of the ATPase of *B. subtilis* using the techniques for protein dissociation in polyacrylamide gel electrophoresis, gives results comparable to those obtained for other bacterial ATPases. After dissociation of purified ATPase of *B. subtilis* by 8 M urea, two bands were observed on polyacrylamide gel electrophoresis, suggesting the presence of two subunits with different charges. With the ATPase of *B. megaterium* KM [9], *S. faecalis* [17] and *M. lysodeikticus* [18], the same result was found.

SDS treatment of ATPase of *B. subtilis* resulted in two very close bands with approximately 1 : 1 ratios during gel electrophoresis, indicating the presence of two subunits with different molecular weights. Using the technique of Swanck and Munkres [16] combining SDS and urea, we observed a better separation of the two bands. The molecular weight of the two subunits was estimated to be about 59 000 (α) and 57 000 (β). This result seems to be very similar to that obtained for *B. megaterium* KM [19] and proteus L forms [14].

In general, the studies in SDS gel electrophoresis, of the subunit structure of bacterial ATPases, reveal two major types of subunits (α and β) with molecular weights of approx. 60 000 and a variable number of minor subunits. As for these minor bands, different results are observed for the same bacteria. Three

minor bands were obtained in *E. coli* NCR 482 [27–29] and K12 [30,31] and two in *E. coli* B [26]. In the case of *M. lysodeikticus*, different solubilization procedures of ATPase by low-salt-wash or by *n*-butanol could be the reason for the presence or absence of minor subunits, respectively [24].

In our first experiments, more than two bands were also obtained, but after introducing the protease inhibitor, during all the solubilization and purification steps, we obtained an enzyme with a specific activity of 20—40 units/mg protein, which yielded only two constant bands during SDS-gel electrophoresis. We believe that in the case of *B. subtilis*, exoprotease and/or membrane proteases are responsible for ATPase degradation and, consequently, it is mandatory to inhibit this proteolytic activity. Abrams et al. [32] obtained an inactive protein from *S. faecalis* and thought of a possible action of cellular proteases on the ATPase. However, phenylmethyl sulfonyl fluoride added during preparation failed to prevent the appearance of this inactive fragment.

Recent works concerning *S. faecalis* [33], *E. coli* [29] and *Thermophilic bacterium* PS3 [34] show the ATPase to be composed of five subunits. One must point out the striking similarity of the molecular weight ($\simeq$35 000) of the γ subunit of these ATPases and of the ATPase from *M. lysodeikticus* [18] although little is known about its function in bacteria [35]. We believe that for other minor subunits it would be very interesting to use phenylmethyl sulfonyl fluoride or another strong protease inhibitor like diisopropylfluorophosphate to show that these fragments are not the results of a limited proteolysis of major subunits. Differences between various bacterial ATPases could reflect a functional specificity related to the bacterial strains but could also stem from structural alterations due to the solubilization and purification procedure.

The results obtained in the enzymic characterization of soluble ATPase of *B. subtilis* may be compared with the other bacterial ATPases already studied. Soluble ATPase of *B. subtilis* requires a divalent cation for activity. The enzyme shows a maximal activity with Ca^{2+} or Zn^{2+}. Mg^{2+} strongly inhibits the activity of the Ca^{2+}-dependent ATPase. The responses of the ATPases to divalent cations appear to depend on the bacterial species. However, the ATPases of *B. megaterium* [3] and *M. lysodeikticus* [36] show a similar behaviour. The fact that three bacteria with closely resembled structural organizations present similar responses, suggests a linkage between ATPase activation by a specific cation and the membrane structure, where the cations participate in its structure and stabilization.

As for all bacterial ATPases, *B. subtilis* ATPase exhibits substrate specificity for purine nucleoside triphosphate. Adenosine nucleotide triphosphate is the most specific substrate of the enzyme but GTP is also hydrolyzed. The K_m for ATP is 1.3 mM, a value close to those obtained for ATPases of *B. megaterium* [3] and of *Rhodospirillum rubrum* [37].

N,N-Dicyclohexylcarbodiimide inhibits the ATPase of *B. subtilis* only in its membrane-bound state, as in the case for the ATPase of *S. faecalis* [38,39] and *E. coli* [40,41]. More complete details on catalytic properties of *B. subtilis* ATPase (BF_1) will be discussed in the second part of this work.

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