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ALTERNATIVE PURIFICATION OF THE MEMBRANE-BOUND ATPase FROM *BACILLUS MEGATERIUM* KM, AND SOME PROPERTIES

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SUMMARY

1. ATPase from the cytoplasmic membrane of *Bacillus megaterium* KM has been purified by glycerol gradient centrifugation. The purified enzyme sediments as a single peak in the analytical ultracentrifuge with a sedimentation velocity constant $s_{20,w}$ of 13.6 S. On polyacrylamide gel electrophoresis at pH 8.0 only one band which also has ATPase activity is seen, but when 8 M urea is added to both sample and gel two subunits are seen. On polyacrylamide gel electrophoresis in 0.1 % sodium dodecyl sulfate the protein is inactivated and one band of molecular weight approximately 69000 is seen. After cold inactivation the ATPase shows a different pattern on polyacrylamide gel electrophoresis at pH 8.0.

INTRODUCTION

We have previously solubilized and purified a membrane-bound Ca^{2+}-activated ATPase from the cytoplasmic membrane of *Bacillus megaterium* KM. The enzyme is released from the membrane by washing extensively with low ionic strength buffer (3 mM Tris–HCl, pH 7.5) which is devoid of divalent cations. In the previous paper we reported a further purification of the enzyme by use of DEAE-cellulose but the final yield was very low[1]. We have therefore devised an alternative purification procedure using glycerol gradient centrifugation which gives a much higher yield and have continued our studies on the properties of the ATPase.

The membrane-bound ATPase from *Streptococcus faecalis* has been isolated and purified. The native enzyme is Mg^{2+}-activated, has a molecular weight of 385000 and seems to consist of twelve subunits of two distinct types, but the same molecular weight of 33000[2,3]. An additional protein, named nectin, of molecular weight 37000 seems to be required for rebinding of purified solubilized ATPase to the depleted membranes. Mg^{2+} is also required[4]. The Ca^{2+}-activated ATPase of *Micrococcus lysodeikticus* cell membranes has been solubilized and partially purified and is estimated to comprise 10 % of the total cytoplasmic membrane protein[5]. Rat liver mitochondrial ATPase which is Mg^{2+}-activated has recently been purified and similarities of molecular weight and amino acid composition exist between the rat liver mitochondrial ATPase and ATPases from beef heart mitochondria, spinach chloroplasts and *S. faecalis*[6]. The mitochondrial ATPase is sensitive to denaturation at 4 °C as is *B. megaterium* KM

ATPase, whereas *S. faecalis* ATPase is not. The membrane-bound ATPases from *B. megaterium* NRLL B929 have been partially purified and studied[7,8].

METHODS

Cultures of *B. megaterium* KM originally obtained from Dr R. Storck were grown overnight in 3 % trypticase soy broth at 30 °C on a New Brunswick rotary shaker. Cells were harvested by centrifuging at 10000 × *g* for 10 min, and were then washed twice with distilled water. Cytoplasmic membranes of *B. megaterium* KM were prepared using lysozyme to remove the cell wall in a hypotonic solution containing deoxyribonuclease but no added Mg^{2+} under conditions outlined in previous experiments[9].

Release of ATPase from cytoplasmic membranes. The membrane-bound ATPase was released by first washing the membranes with 0.03 M Tris–HCl, pH 7.5 (3 times) followed by further washing with 0.003 M Tris–HCl, pH 7.5 (twice) as described previously[1]. The high-activity ATPase released from the membrane in the last two washes was pooled, ribonuclease was added to a final concentration of 1 μg/ml and the solution was precipitated with solid $(NH_4)_2SO_4$ to 80 % saturation. It was stored in the refrigerator until ready for use.

Purification. The $(NH_4)_2SO_4$ precipitate was centrifuged at 27000 × *g* for 10 min and the supernatant discarded. The precipitate was resuspended in 0.003 M Tris–HCl, pH 7.5, and dialysed against several changes of 0.03 M Tris–HCl, pH 7.5, over a period of at least 2 h. This sample was then centrifuged at 39000 × *g* for 15 min to get rid of any insoluble material and the clear yellowish solution was layered on to 7–30 % glycerol gradients containing 0.03 M Tris–HCl, pH 7.5. The tubes were spun in a Beckman L2-65B ultracentrifuge for 24 h at 16 °C at 27000 rev./min using large tubes in the SW 27 rotor. Fractions of 2 ml were collected from the top of the tube and the absorbance at 280 nm monitored continuously using a modified Zeiss spectrophotometer. Each fraction was tested for ATPase activity. The fractions containing ATPase activity were pooled, precipitated with $(NH_4)_2SO_4$ to 80 % saturation and stored in the refrigerator. The membranes used in experiments in which rebinding of the solubilized ATPase to depleted membranes was studied were those obtained by extensive washing.

Assay of enzyme activity. A unit of activity is defined as that amount of protein which liberates 1 μmole P_i in 10 min at pH 7.5 and 37 °C. Specific activity is expressed as units/mg. Protein was determined either by the Biuret method[10] or by the method of Lowry *et al.*[11]. ATPase activity was determined by measuring release of P_i into a medium containing 0.01 M Tris–HCl, pH 7.5, 0.01 M $CaCl_2$, 8 mM ATP, unless otherwise stated, and 1–200 μg protein in a total volume of 2 ml held at 37 °C for 10 min. The reaction was stopped by putting the mixture in ice, and when necessary 0.1 ml of concentrated $HClO_4$ was added to precipitate any protein, which was then removed by centrifugation. The P_i released was measured by the method of Fiske and Subba-Row[12].

Polyacrylamide gel electrophoresis. Polyacrylamide gels containing 0.1 % sodium dodecyl sulfate made by the method of Shapiro *et al.*[13] as described by Weber and Osborn[14] were used. Samples were heated with 0.1 % sodium dodecyl sulfate, 0.1 % mercaptoethanol for 10 min at 60 °C before running on the gel. Approximate molec-

ular weights were estimated using bovine serum albumin, ovalbumin and cytochrome *c* as molecular weight markers[14]. We also used a gel system in which 7.5 % polyacrylamide gels containing 0.015 M Tris and glycine titrated to pH 8.0 were used, and gels were stained for protein and ATPase activity as described previously[1].

Ultracentrifuge studies. Sedimentation velocity runs were made in a Beckman Model E ultracentrifuge. Runs were made at 42040 rev./min and a temperature of 20 °C, at ATPase concentrations between 1 and 2 mg/ml, in 0.03 M Tris–HCl, pH 7.5.

RESULTS

As reported previously, the specific activity of the ATPase released from the membranes by extensive washing is generally between 40–60 units, as compared with 1–4 units in the cytoplasmic membrane itself[1]. Fig. 1 shows a typical sedimentation profile of protein and ATPase activity obtained after glycerol gradient centrifugation. A single protein peak corresponds closely with the single peak of ATPase activity. When the fractions (10 and 11) containing ATPase of high specific activity are pooled and re-run on the glycerol gradient, a symmetrical peak of both protein and ATPase activity is obtained. The specific activity of the doubly purified ATPase is between 360–600 units compared with 450–1000 units for ATPase purified on DEAE-cellulose[1]. However, we suspect that this lower average value for the specific activity reflects loss of activity rather than lack of purity since we see a single band on polyacrylamide gel electrophoresis and we know the enzyme is extremely sensitive to denaturation. The specific activity through the peak in three runs gave an average specific activity of 473 units and 455 units in the two tubes comprising the major portions of the peak. The ultraviolet spectrum of the purified ATPase shows a maximum at 276 nm and a

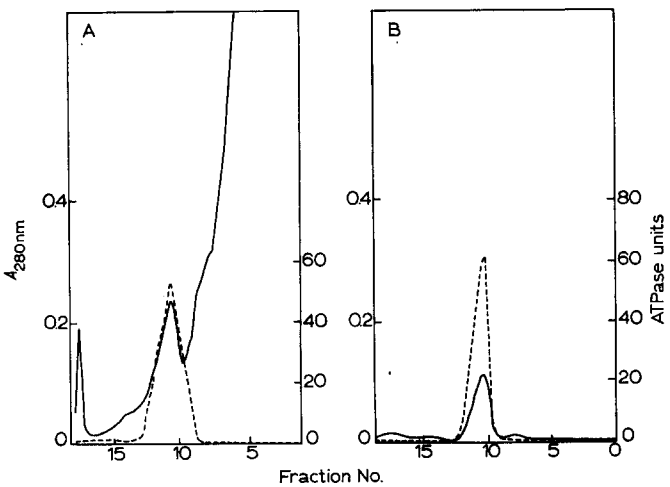

Fig. 1. Purification of membrane ATPase by glycerol gradient centrifugation. 2-ml fractions of $(NH_4)_2SO_4$-precipitated ATPase were layered on to 7–30 % glycerol gradients containing 0.03 M Tris–HCl, pH 7.5. Tubes spun at 27000 rev./min for 24 h at 16 °C using large tubes in the SW27 rotor. Fractions collected from top of tube and absorbance at 280 nm monitored continuously. A. profile of fraction released from cytoplasmic membranes. B. ATPase isolated from A and recentrifuged.

minimum at 250 nm. The yield of ATPase starting with a wet weight of bacteria of 60 g is between 10–20 mg protein for the material run once on the glycerol gradient.

Sedimentation velocity runs of the purified ATPase in the analytical ultracentrifuge showed a single symmetrical peak with an average $s_{20,w}$ of 13.6 S (three runs) (see Fig. 2). Polyacrylamide gel electrophoresis of the purified protein using 7.5 % gels

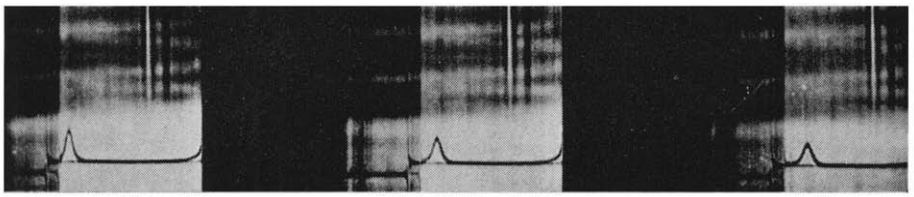

Fig. 2. Sedimentation velocity profile of purified ATPase in Beckman Model E analytical ultracentrifuge. ATPase (2 mg/ml) dialysed against 0.03 M Tris–HCl, pH 7.5, was placed in a double sector cell and sedimented at 42 040 rev./min and 20 °C. Photographs taken at 4-min intervals. Bar angle setting 80°. Sedimentation coefficients determined by measuring the maximum ordinate in the Schlieren patterns with a microcomparator.

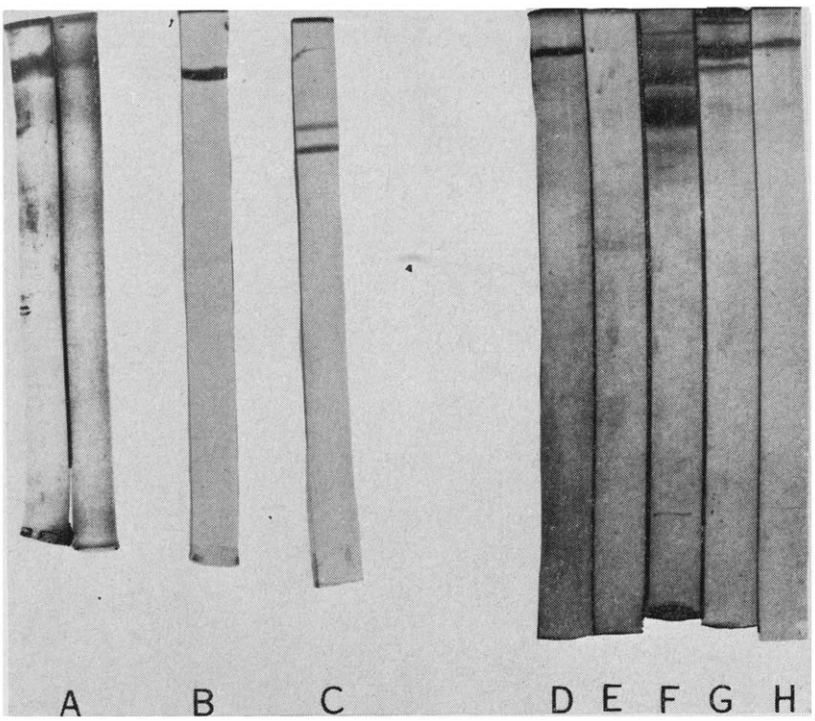

Fig. 3. Polyacrylamide gel electrophoresis at pH 8.0, Tris–glycine gels 2–50 μg sample layered on to each gel. Gels run at 210 V until bromophenol blue dye reached bottom of gel (approximately 1.5 h). A, B, C, purified ATPase; A, gel stained for protein; gel stained for ATPase as described in Methods; B, gel stained for protein with Coomassie brilliant blue; C, purified ATPase dissolved in 8 M urea, 0.1 % β-mercaptoethanol run in Tris–glycine gel contaning 8 M urea; D–H, tubes from different portions of glycerol gradient; D, ATPase fraction (Tubes 10 + 11); E, Tube 1; F, Tube 9; G, Tube 13; H, tube 17.

and Tris–glycine buffer at pH 8.0 shows a single major band with traces of minor bands when the gels are overloaded with respect to ATPase. All of the ATPase activity is found in the major band, and protein samples from other portions of the glycerol gradient show different band patterns, although samples from the bottom of the gradient may contain denatured ATPase (see Fig. 3). The major band is in an identical position to the band found using ATPase purified using DEAE-cellulose and on polyacrylamide gel electrophoresis we also find, as reported previously, a single subunit of molecular weight 69 000[1]. Polyacrylamide gel electrophoresis of the purified ATPase in Tris–glycine gels at pH 8.0 containing 8 M urea reveals two bands, suggesting the presence of two subunits of different charge (Fig. 3).

Previous studies showed that the purified ATPase was inactivated when incubated at 4 °C. We therefore incubated a sample at 4 °C for 8 h and 48 h and compared the band patterns obtained on polyacrylamide gel electrophoresis with that of a control sample (see Fig. 4). There is a striking difference in the band patterns, most of the band normally associated with ATPase activity being converted to a much faster moving component. This can be seen very clearly in the profile obtained after 48 h where almost complete disappearance of the ATPase band occurs, simultaneously with the appearance of the faster moving band suggesting that conversion of the native enzyme into another form is occurring. Sedimentation velocity runs of cold-inactivated ATPase in the analytical ultracentrifuge show the presence of a main peak having an $s_{20,w}$ value of 13.6 S, with other species of higher $s_{20,w}$ values present as well.

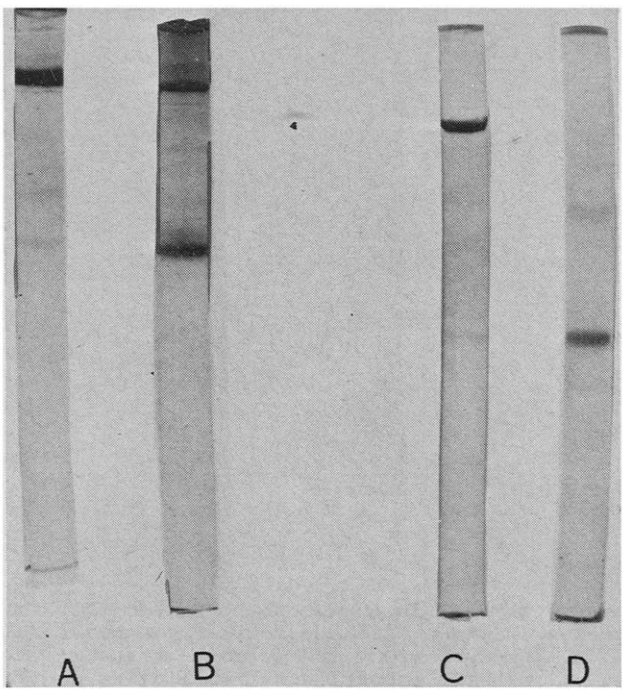

Fig. 4. Polyacrylamide gel electrophoresis at pH 8.0, Tris–glycine gels. A and C, purified ATPase; B, ATPase held at 0 °C for 8 h; D, ATPase held at 0 °C for 48 h.

DISCUSSION

Purification of the Ca^{2+}-activated membrane ATPase of *B. megaterium* KM using glycerol gradient centrifugation results in higher yields than the alternative method using DEAE-cellulose[1]. The absorbance profile at 280 nm of the glycerol gradient, the results obtained on polyacrylamide gel electrophoresis, and the symmetrical sedimentation velocity profile indicate that a highly purified ATPase has been obtained. The $s_{20,w}$ value of 13.6 S compares closely with a value of 13.4 S obtained for the purified ATPase of *S. faecalis*[3]. The ATPase from *B. megaterium* KM purified by either method seems to split into subunits of molecular weight approximately 69000 as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, in contrast with the subunit weight of 33000 calculated from ultracentrifugation in 6 M guanidine hydrochloride for the *S. faecalis* ATPase. On polyacrylamide gel electrophoresis in 8 M urea we see two distinct subunits as do Schnebli and Abrams[2] with *S. faecalis* ATPase. Although mitochondrial ATPase has the same molecular weight and similar amino acid composition to the *S. faecalis* enzyme subunit weights of 62500, 57000 and 36000 are reported for this enzyme[6]. However, considerable discrepancies exist between different published values of the molecular weight of the mitochondrial enzyme and its subunits. Initial studies indicated a molecular weight of 284000 for the native enzyme, with a subunit weight of approximately 26000[15]. More recently four different classes of subunits have been reported using sodium dodecyl sulfate polyacrylamide gel electrophoresis[16] and a study using 6 M guanidine hydrochloride to separate subunits obtained and subunit molecular weight of 46000 in the analytical ultracentrifuge[17]. The cold inactivation of the mitochondrial ATPase parallels the formation of subunits in this enzyme, as shown by a decrease in sedimentation velocity coefficient from 12.9 S for the native enzyme to 3.5 S on complete cold inactivation[15]. The altered mobility on polyacrylamide gel electrophoresis of the *B. megaterium* KM ATPase which has been cold-inactivated probably reflects a conformation change of some kind. We have been unable to observe a decrease in sedimentation velocity coefficient with cold-inactivated ATPase, and although we observe the presence of heavier species than the native ATPase, the main peak seen in the ultracentrifuge on cold inactivation has an $s_{20,w}$ value almost identical with that of the native enzyme. Our results indicate that we have a highly purified ATPase from the cytoplasmic membrane of *B. megaterium* KM, and we are continuing our investigation of the chemical and physical properties of the enzyme and its relationship to the whole cytoplasmic membrane.

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