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ANTIBODIES TO PURIFIED MEMBRANE-BOUND ATPase FROM *BACILLUS MEGATERIUM* KM AND THEIR REACTION WITH PROTOPLASTS AND CYTOPLASMIC MEMBRANES

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SUMMARY

Antibodies to the solubilized purified Ca^{2+}-activated ATPase from the cytoplasmic membrane of *Bacillus megaterium* KM form a single precipitin line when they are tested against the homologous antigen. The antibodies inhibit both soluble and membrane-bound ATPase activity. The inhibition is non-competitive. Both protoplasts and cytoplasmic membranes of *B. megaterium* KM can compete with soluble ATPase for antibody although membranes compete more effectively than protoplasts. Addition of anti-ATPase immunoglobulin (IgG) to protoplasts or membranes causes agglutination. No agglutination occurs with control IgG. The clumping can be prevented by addition of purified ATPase to the IgG before mixing with the protoplasts or membranes. These results suggest that part of the ATPase molecule may be exposed on the outer surface of the cytoplasmic membrane, and part on the inner surface.

INTRODUCTION

We have previously reported the purification, molecular weight, amino acid composition and other properties of the Ca^{2+}-activated ATPase from the cytoplasmic membrane of *Bacillus megaterium* KM [1–3]. The ATPase from *Micrococcus lysodeikticus* cytoplasmic membrane has been solubilized and partially purified [4] and antibody to both the purified protein [5] and the cytoplasmic membrane has been made [6]. The localization and distribution of the membrane bound ATPase has also been investigated using ferritin labelled antibodies to the ATPase [7].

Since we are interested in the interaction of the solubilized ATPase with the membrane, and its spatial location in vivo, we decided to investigate the reactions of antibodies made against purified solubilized ATPase with solubilized ATPase, and also with protoplasts and cytoplasmic membranes from *B. megaterium* KM.

MATERIALS AND 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 10 000 × *g* for 10 min and were then washed twice with distilled water. Protoplasts were prepared by digesting the cell wall with lysozyme, at a final concentration of 0.4 mg/ml in a buffer containing 0.6 M sucrose, 0.03 M Tris–HCl, pH 7.5, and 0.01 M $CaCl_2$. Protoplast formation was followed by phase-contrast microscopy. An estimate of the percentage of protoplasts which had lysed was made by comparing the absorbance at 260 nm of the supernatant from a sample of intact protoplasts centrifuged at 27 000 × *g* for 10 min with the supernatant from a similar sample of protoplasts burst by diluting them with a solution of 0.03 M Tris–HCl, pH 7.5, containing 1 μg/ml deoxyribonuclease [8]. Cytoplasmic membranes were prepared under conditions outlined previously [1]. Membranes were washed twice with 0.03 M Tris–HCl, pH 7.5 prior to use.

Bacillus subtilis (strain Marburg) was grown overnight on 3% trypticase soy broth. Membranes were made under the same conditions used for preparation of the *B. megaterium* KM membranes.

Purification of ATPase

Membrane-bound ATPase was released from the cytoplasmic membrane by first washing three times with 0.03 M Tris–HCl (pH 7.5) followed by two further washes with 0.001 M Tris–HCl (pH 7.5). ATPase released in the last two washes was then purified by centrifugation in a 7–30% glycerol gradient as described previously [2].

Assay of enzyme activity

ATPase activity was estimated as described previously [1].

Immunizations

Two 6-month-old, male New Zealand White rabbits were immunized intramuscularly in the thigh and intradermally at several sites along the back. Two series of immunizations were administered 10 days apart. Three booster injections were given at 10 day intervals after the second immunization. The first two series of injections were made with a 1-ml solution of 5 mg/ml ATPase dialyzed against 0.15 M NaCl, mixed with 1 ml of Freund's complete adjuvant, for each rabbit. Booster injections were made with 1 ml of 4 mg/ml ATPase in 0.15 M NaCl. Rabbits were bled twice through the ear vein, 10 days after the first booster injection, and 8 days after the third booster injection. The γ-globulin (IgG) fraction was obtained by fractionation with 33% $(NH_4)_2SO_4$. The ATPase used for these immunizations had been purified once by glycerol gradient centrifugation. It has a specific activity of approximately 300 units (μmoles P_i in 10 min at pH 7.5, 37 °C), and appears as a single line on polyacrylamide gel electrophoresis in Tris–glycine gels at pH 8.0 [2]. A third rabbit was immunized with ATPase of specific activity of approximately 600 units.

Micro-Ouchterlony plates were made as described by Williams and Chase [9] using 1% Difco Noble agar in 0.01 M phosphate, 0.15 M NaCl. Anti-serum or anti-IgG was placed in the center well, test solutions in the surrounding wells. Plates were incubated at room temperature for 48 h, washed for 24 h against two changes of 1% NaCl, rinsed for 2 h in distilled water, left to dry and then stained with Coomassie brilliant blue.

Inhibition of ATPase activity with anti-ATPase IgG

Purified ATPase was incubated for 20 min at 37 °C with varying quantities of anti-ATPase IgG and also with a control rabbit IgG. ATP was then added to the mixture to start the ATPase assay which was carried out in the normal way. In some inhibition experiments constant quantities of IgG and ATPase were used and the ATP concentration was varied. In these experiments enough IgG was used so that the ATPase activity was inhibited between 30–80% at maximum substrate concentration. In another experiment we added ATP at a final concentration of $2 \cdot 10^{-4}$ M to the purified ATPase before incubating with varying quantities of anti-ATPase IgG.

In experiments with cytoplasmic membranes and protoplasts only freshly prepared membranes and protoplasts were used.

Competition of membranes and protoplasts for anti-ATPase IgG

To test whether the membranes and protoplasts would compete with purified soluble ATPase in binding IgG, we first diluted the anti-ATPase IgG five times with 0.03 M Tris–HCl, pH 7.5, or 0.03 M Tris–HCl, 0.6 M sucrose, 10 mM $CaCl_2$, pH 7.5, and determined the quantity of this IgG which would inhibit a given sample of ATPase by approximately 50%. This quantity of antibody was then incubated with varying amounts of freshly prepared membranes or protoplasts for 20 min at room temperature. Then the solutions were centrifuged at 27 000 $\times g$ for 10 min so that the protoplasts or membranes were completely sedimented. The IgG supernatant was incubated with ATPase for 20 min at 37 °C as described above and the ATPase activity of the solution determined. A control of the ATPase activity of the supernatant serum was included.

Agglutination experiments

A sample of serum, or IgG diluted with 0.03 M Tris–HCl, pH 7.5, for experiments with membranes or with 0.03 M Tris–HCl, pH 7.5, 0.6 M sucrose, 10 mM $CaCl_2$ for experiments with protoplasts to a final protein concentration of between 0.02 and 1 mg/ml was mixed with an equal volume of either membranes or protoplasts and allowed to stand at least 5 min. 10 μl of the mixture was then put on a microscope slide and observed in a Zeiss phase-contrast microscope with an oil-immersion lens under 1250 $\times$ magnification. In some experiments ATPase solution or ATP, or both, was added to the serum and allowed to stand at room temperature for at least 20 min before mixing with the membranes or protoplasts.

Precipitin ring test

This was performed in 10-μl disposable pipets. 5 μl of undiluted serum was tested against 5 μl of purified ATPase (1 mg/ml) in 0.03 M Tris–HCl, pH 7.5, diluted 1, 10, 100 and 1000 times.

RESULTS

The rabbits made antibodies to the soluble, purified Ca^{2+}-activated ATPase from *B. megaterium* KM. In the precipitin ring test precipitates were seen in the tubes containing the 1, 10 and 100 times diluted ATPase within 1.5 h. A single precipitin line was observed in Ouchterlony plates between the ATPase and both anti-serum

and anti-ATPase IgG. The IgG can completely inhibit the enzyme activity of both the purified soluble ATPase and also membrane-bound ATPase. 1 mg of soluble purified ATPase was inhibited by 36.4 mg of IgG protein, equivalent to 3.22 ml of IgG, and 1 mg of cytoplasmic membranes in 80% inhibited by 0.725 mg of IgG protein. The inhibition of soluble ATPase activity by IgG is non-competitive (see Fig. 1). In experiments where we added ATP prior to incubation of ATPase with IgG, the ATP did not seem to exert a protective effect, and inhibition was identical with that of samples without added ATP.

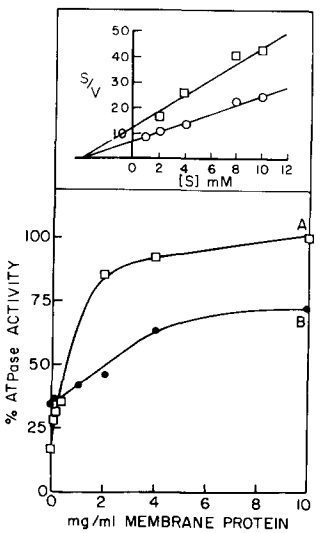

Fig. 1. Top: Inhibition of the ATPase activity by the anti-ATPase IgG in the presence of substrate. □–□, purified ATPase incubated with anti-ATPase IgG for 30 min at 37 °C prior to addition of Ca^{2+} and ATP (in 1.25:1 ratio). Concentrations of enzyme and IgG were adjusted to give approximately 50% inhibition at the highest substrate concentration. ●–●, purified ATPase incubated with control rabbit IgG prior to addition of ATP and Ca^{2+}. Bottom: Relief of inhibition of ATPase by IgG. □–□, IgG incubated with cytoplasmic membranes for 20 min at 25 °C prior to incubation with purified ATPase (see Materials and Methods). ●–●, IgG incubated with protoplasts for 20 min at 25 °C prior to incubation with purified ATPase.

In experiments comparing the properties of protoplasts with those of cytoplasmic membranes it is important to determine the percentage of protoplasts which have lysed on treatment of *B. megaterium* KM cells with lysozyme in the presence of sucrose. Weibull [10] reported that protoplasts were stabilized by the addition of 0.005–0.01 M Mg^{2+} to the sucrose medium, and we found that we could substitute 0.01 M Ca^{2+} for Mg^{2+} as a stabilizing agent. In these experiments approximately 88% of the protoplasts remained intact as estimated by a comparison of optical densities at 260 nm of the supernatants from intact and lysed protoplasts [8]. Protoplasts exhibit a small but variable amount of ATPase activity, 40% of which is susceptible to inhibition by 10^{-5} M dicyclodihexocarbodiimide, an inhibitor which inhibits a comparable sample of membrane-bound ATPase from *B. megaterium* KM up to 80% at a concentration of 10^{-5} M. (Unpublished experiments.)

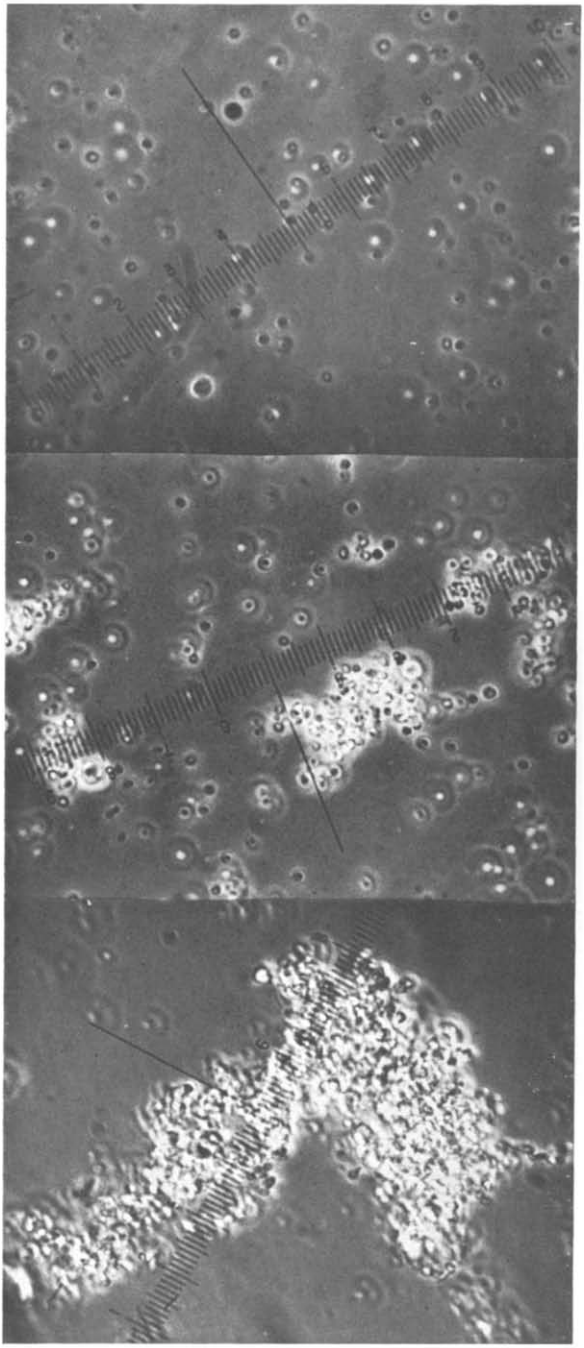

Fig. 2. Agglutination of protoplasts by anti-ATPase IgG. Top: Protoplasts plus control rabbit IgG. Center: Protoplasts plus anti-ATPase serum. Bottom: Protoplasts plus anti-ATPase IgG. Large and small clumps are seen both with serum and IgG.

To test whether either protoplasts or membranes could bind to antibodies made against the purified soluble ATPase, we incubated varying amounts of either protoplasts or membranes with anti-ATPase IgG. We then sedimented the protoplasts or membranes and tested the ability of the supernatant IgG to inhibit purified soluble ATPase. We found that both protoplasts and membranes could compete with the soluble ATPase for antibody, since IgG that had previously been incubated with either protoplasts or membranes was less inhibitory than control anti-ATPase IgG. As increasing quantities of both protoplasts and membranes are used, the ATPase inhibition by anti-ATPase IgG is decreased (see Fig. 1). Protoplasts are about 22% as effective in relieving inhibition as membranes. Since we estimate from a comparison of optical densities at 260 nm that only 12% of the protoplasts had burst after centrifugation, the results cannot be explained on the basis of burst protoplasts alone.

If anti-ATPase IgG or whole serum is mixed with a suspension of protoplasts or cytoplasmic membranes at room temperature agglutination occurs over a period of several minutes (see Fig. 2). No clumping is seen with a non-specific rabbit IgG, or an addition of an equal quantity of sucrose–Tris–$CaCl_2$ solution. The agglutination occurs very slowly at 4 °C, although protoplasts left overnight at 4 °C in the presence of serum will agglutinate. If a mixture of protoplasts and anti-ATPase IgG originally incubated at 4 °C is warmed up, then agglutination occurs over a period of about 20 min. We found that if protoplasts were diluted to a volume equivalent to 20 times the net weight of the bacteria from which they were made, a 100-fold dilution of serum still causes appreciable clumping (i.e. 0.065 mg IgG will agglutinate 1 mg of cytoplasmic membranes). The agglutination of either protoplasts or membranes could be prevented by adding soluble purified ATPase to the serum before mixing (serum diluted 50 times, containing 0.23 mg/ml protein plus 0.25 mg/ml ATPase) as described in Materials and Methods. Addition of ATP to serum before mixing has no effect on agglutination. Both the serum made with once purified ATPase, and serum made against ATPase of the highest purity showed the agglutination reaction to a comparable extent. Membranes of *B. subtilis* did not agglutinate in the presence of IgG prepared against the ATPase from *B. megaterium* KM.

DISCUSSION

The agglutination of protoplasts by both anti-ATPase IgG and serum suggests that a part of the ATPase molecule may be exposed to the outer surface of the membrane so that binding to the IgG can occur. Since antibodies prepared against ATPase showing only two lines on sodium dodecylsulfate polyacrylamide gel electrophoresis, and therefore of the highest purity, showed the agglutination reaction, it is extremely unlikely that this result is due to impurities present in the ATPase preparation. The competition for anti-ATPase IgG between purified ATPase and protoplasts supports this suggestion. The fact that protoplasts only show small amounts of ATPase activity compared with membrane ATPase activity, and that the membranes compete more effectively than protoplasts for IgG might also indicate that only a small portion of the molecule is exposed on the outer surface of the membrane. Whiteside and Salton [5] and Fukui et al. [6] report that in *M. lysodeikticus*, protoplasts do not absorb out antibody to the purified soluble ATPase of the cytoplasmic membrane, and localization studies with ferritin labelled antibodies also suggest that the ATPase must be

located on the inner surface of the membrane [7]. However, they did not test for agglutination or for competition between protoplasts and soluble ATPase for serum. They did find that an antigen specific to ATPase treated with 0.1% sodium dodecylsulfate was absorbed by protoplasts. Studies on the interaction of solubilized membrane ATPase from *Streptococcus faecalis* with lipid bilayer membranes suggest that the ATPase interacts with the lipid bilayer to form relatively stable conducting channels in the membrane probably in the form of aqueous-filled pores [11]. Therefore, it seems possible that the ATPase of *B. megaterium* KM spans the membrane, though further experiments are needed to prove this conclusively.

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