BBA 75732

PURIFICATION AND PROPERTIES OF ATPase FROM THE CYTOPLASMIC MEMBRANE OF *BACILLUS MEGATERIUM* KM

RHONA MIRSKY AND VIRGINIA BARLOW
Department of Biochemistry, Dartmouth Medical School, Hanover, N.H. 03755 (U.S.A.)
(Received April 26th, 1971)

SUMMARY

1. ATPases are found associated with a wide variety of cell membranes. We have solubilized and purified a membrane-bound ATPase from the cyotplasmic membranes of *Bacillus megaterium* KM.

2. The properties of the membrane bound and solubilized enzyme are similar. It is activated by both Ca^{2+} and Mg^{2+}, but preferentially by Ca^{2+}. It is slowly inactivated at 4°. In addition to ATP it can use GTP and ITP as substrates. It is competitively inhibited by ADP.

3. The solubilized ATPase binds back to membranes depleted of ATPase, in the presence of 0.01 M Ca^{2+}. It does not bind to undepleted membranes.

4. The enzyme is released from the membrane by washing with 0.003 M Tris–HCl (pH 7.5). It is further purified by absorption on DEAE-cellulose, followed by elution with $(NH_4)_2SO_4$. On polyacrylamide gel electrophoresis at pH 8.8 and pH 8.0, the purified enzyme appears as a major band which has ATPase activity. On polyacrylamide gel electrophoresis in 0.1 % sodium dodecyl sulfate, the protein is inactivated and one major band of molecular weight approx. 69000 is seen. Since the active protein is excluded from Sephadex G-100 gel, its molecular weight must be greater than 100000, suggesting that the band seen on polyacrylamide gel electrophoresis in sodium dodecyl sulfate must represent subunits of the whole protein.

INTRODUCTION

This study aims at a further understanding of the cytoplasmic membrane proteins of *Bacillus megaterium* KM. Previously we have isolated an insoluble membrane protein fraction from the cytoplasmic membranes[1]. We have also isolated and purified flagellin from *B. megaterium* KM and shown that it is one of the major proteins seen on polyacrylamide gel electrophoresis of whole cytoplasmic membranes even when flagella are removed prior to isolating the membranes.

ATPases (ATP phosphohydrolases, EC 3.6.1.3) are found associated with a wide variety of membrane systems and the bacterial membrane-bound ATPases studied have some properties in common with each other and with the oligomycin sensitive mitochondrial ATPase[3,4] but substantial differences exist, too. Three bacterial membrane-bound ATPases have been studied in some depth. ABRAMS[5] and ABRAMS AND BARON[6,7] solubilized and purified membrane-bound ATPase from *Streptococcus*

faecalis and studied its properties and reversible attachment to the membrane. They estimated that it comprised 2 % of the total cytoplasmic membrane protein. The ATPase of *Micrococcus lysodeikticus* cell membrane has been solubilized and partially purified[8] and is estimated to comprise 10 % of the total cytoplasmic membrane protein. Antibody to the purified protein has also been made[9]. The membrane-bound ATPases of *B. megaterium* NRLL B929 have been partially purified and studied[10,11]. Two peaks of activity having similar properties are found even in the purest preparations.

We have solubilized and substantially purified the ATPase associated with the cytoplasmic membrane of *B. megaterium* KM. We find a single ATPase which is activated by Ca^{2+} and Mg^{2+}, with a preference for Ca^{2+}, which is cold sensitive, specific for purine trinucleotides and competitively inhibited by ADP.

METHODS

Cultures of *B. megaterium* KM originally obtained from Dr. R. Storck were grown overnight in 3 % trypticase soy broth at 30° 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[1].

Release of ATPase from cytoplasmic membranes

ATPase activity was released from the membranes using a modification of the procedure of ABRAMS[5]. Membranes were sedimented at 39000 × *g* for 50 min (Stage 1). They were resuspended in 0.03 M Tris–HCl, pH 7.5 (10 ml buffer/g wet weight of original bacterial suspension) and centrifuged at 39000 × *g* for 30 min (Stage 2). This procedure was repeated twice more using 3 ml buffer/g wet weight of original bacterial suspension (Stages 3 and 4). The membranes were then resuspended in 0.003 M Tris–HCl, pH 7.5 (3 ml buffer/g wet weight of original bacterial suspension) and centrifuged at 39000 × *g* for 30 min. The supernatant from this stage contained high specific activity ATPase and was saved for further purification (Stage 5). The washing procedure was repeated twice more using 0.003 M Tris–HCl, pH 7.5 (1.5 ml buffer/g wet weight of original bacterial suspension) and the supernatant from Stage 6 was saved for further purification. The membranes used in experiments on membrane bound ATPase activity were those obtained by this extensive washing (Stage 8). They were also used for the experiments in which the rebinding of the solubilized ATPase to depleted membranes were studied. For studies of rebinding of ATPase to undepleted membranes we used membranes which had been washed twice with 0.03 M Tris–HCl after lysis of the whole cells.

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°. Specific activity is expressed as units/mg of protein. Protein was determined either by the Biuret method[12] or by the method of LOWRY *et al.*[13]. 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 $CaCl_2$, 0.008 M ATP unless otherwise stated,

and 1–200 μg protein in a total volume of 2 ml held at 37° for 10 min. The reaction was stopped by putting the mixture in ice, and when necessary 0.1 ml of concentrated perchloric acid was added to precipitate any protein, which was then removed by centrifugation. 0.5 ml of supernatant were then used to measure the P_i released, by the method of FISKE AND SUBBAROW[14].

Polyacrylamide gel electrophoresis

Polyacrylamide gels containing 0.1 % sodium dodecyl sulfate made by the method of SHAPIRO *et al.*[15] as described by WEBER AND OSBORN[16] were used. Samples were heated with 0.1 % sodium dodecyl sulfate, 0.1 % mercaptoethanol for 3 min at 100° before running on the gel. Bromophenol blue was used to mark the solvent front. This method was also used to estimate approximate molecular weights[16]. Bovine serum albumin, ovalbumin and cytochrome *c* were used as molecular weight markers. We also used a system in which 7.5 % polyacrylamide gels containing 0.015 M Tris and glycine titrated to varying pH values were used. The gel and tank buffers were the same and the gels were run at a current of 2 mA/tube for about 45 min. Gels were stained for protein using Coomassie brilliant blue. ATPase activity in the gels was detected by immersing for 30 min at 37° in a solution containing 0.005 M $CaCl_2$, 0.1 M Tris–HCl (pH 7.5), 0.008 M ATP, followed by staining in a solution containing the standard proportions of H_2SO_4, $(NH_4)_2MoO_4$ and reducing agent used in the Fiske–SubbaRow assay.

Reagents

All reagents used were analytical grade.

RESULTS

Release of ATPase from the cytoplasmic membrane

By using a modification of the procedure of ABRAMS[5], we have obtained a highly selective release of membrane bound ATPase from the cytoplasmic membranes of *B. megaterium* KM (see Table I). Release of ATPase with high specific activity always

TABLE I

RELEASE OF ATPase FROM CYTOPLASMIC MEMBRANES OF *B. megaterium* KM

Procedure and assay conditions described in text.

Washing procedure	*Buffer*	*ATPase specific activity (units/mg protein)*
1. Lysate from lysozyme treatment	0.03 M Tris–HCl, pH 7.5	1.7
2. Supernatant from Stage 1	0.03 M Tris–HCl, pH 7.5	0.6
3. Supernatant from Stage 2	0.03 M Tris–HCl, pH 7.5	4.0
4. Supernatant from Stage 3	0.03 M Tris–HCl, pH 7.5	3.5
5. Supernatant from Stage 4	0.03 M Tris–HCl, pH 7.5	2.3
6. Supernatant from Stage 5	0.003 M Tris–HCl, pH 7.5	40.0
7. Supernatant from Stage 6	0.003 M Tris–HCl, pH 7.5	53.0
8. Supernatant from Stage 7	0.003 M Tris–HCl, pH 7.5	4.3
9. Depleted cytoplasmic membranes	0.003 M Tris–HCl, pH 7.5	0.9

occurs in the wash in which the buffer concentration is lowered from 0.03 M Tris–HCl (pH 7.5) to 0.003 M Tris–HCl (pH 7.5). Specific activity in this wash (Stage 5) was normally lower than in the subsequent wash (Stage 6), but a larger fraction of the ATPase was released into the supernatant at Stage 5. The third wash with 0.003 M Tris–HCl (Stage 7) normally contains a very small quantity of ATPase activity. However, the washed cytoplasmic membranes retain considerable ATPase activity. In an experiment comparing the recovery of total ATPase units with recovery of protein throughout the various stages of this procedure, we accounted for 85 % of the total ATPase units and 93 % of the total protein.

Effects of Ca^{2+} and Mg^{2+}

There was no activity in either membrane bound or soluble preparations when no divalent ions were present. Addition of either Ca^{2+} or Mg^{2+} activated the enzyme but the extent of activation was always greater with Ca^{2+} than Mg^{2+} at pH 7.5 (Fig. 1).

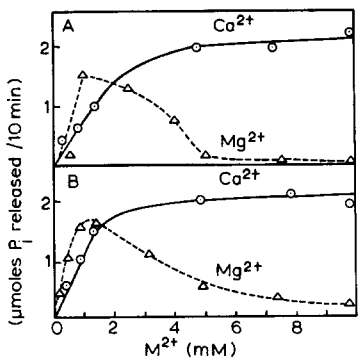

Fig. 1. Effect of Ca^{2+} and Mg^{2+} on velocity of ATP hydrolysis by solubilized and membrane bound ATPase from *B. megaterium* KM. ATPase activity measured as described under METHODS. Solubilized ATPase used was Stages 5 and 6; membranes were washed 7 times as described under METHODS.

Mg^{2+} activates the enzyme at a lower concentration than Ca^{2+} and inhibits at higher concentrations. When Mg^{2+} was added to solutions containing 0.01 M $CaCl_2$, it inhibited the Ca^{2+}-ATPase activity. The percentage activation obtained with Mg^{2+} varied considerably in different preparations of both membrane bound and solubilized preparations. In three preparations of membrane bound ATPase the percentage activation Mg^{2+}/Ca^{2+} was 35, 47 and 82 %, and for the soluble ATPase Mg^{2+}/Ca^{2+} activation varied from 10 to 80 %. There was no activation or stimulation of ATPase activity by Na^+ or K^+.

Cold inactivation of ATPase activity

When both the solubilized and membrane bound enzymes were incubated in 0.003 M Tris–HCl (pH 7.5) at 4°, the enzyme was slowly inactivated (Table II). The rate of inactivation for the solubilized enzyme depends on concentration with more concentrated solutions being better protected against the cold. At room temperature, solubilized and membrane bound ATPase normally retained full activity for about

TABLE II

COLD INACTIVATION OF ATPase

Samples were left in 0.003 M Tris–HCl (pH 7.5) at either 23° or 4° for the periods of time specified. Samples were withdrawn and assayed for ATPase activity as described in the text.

Sample	*Time* (*h*)	*Inactivation of ATPase* (%)	
		23°	*4°*
Membranes washed 6 times	0	0	0
	4	0	50
	6	0	70
	8	0	68
	24	0	92
Supernatant containing ATPase activity	0	0	0
	2	0	0
	4	0	41
	6	0	60
	24	0	87

30 h. Addition of ADP at a concentration of $5 \cdot 10^{-4}$ M had no effect on the rate of inactivation in the cold.

The effect of rapid freezing was variable. In some preparations we could freeze and thaw the solubilized enzyme with very little loss of activity. However, on other ocassions the solubilized ATPase lost all activity on freezing. In an experiment in which a 1-ml sample of solubilized enzyme was frozen rapidly in dry ice–ethanol and then thawed and assayed immediately there was a 58 % inactivation compared with the control. Membrane bound ATPase could be frozen with little loss of activity.

Substrate specificity

Ca^{2+}-activated ATPase was activated by ATP in the presence of 0.01 M $CaCl_2$. We found that we could vary the ATP/Ca^{2+} ratio from 1:5 to 1:1 without affecting the activity of the enzyme. Table III shows the substrate specificity of the enzyme.

TABLE III

RELATIVE ACTIVITIES OF SOLUBILIZED ATPase TOWARD VARIOUS SUBSTRATES

Different substrates were incubated with solubilized ATPase in the presence of 0.002 M substrate, 0.01 M $CaCl_2$ or 0.001 M $MgCl_2$, 0.1 M Tris–HCl (pH 7.5) for 10 min at 37°, and were then assayed for P_i as described in the text.

Substrate	*ATPase activity*	
	Mg^{2+} (expressed as % Ca^{2+} activity)	*Ca^{2+}* (%)
ATP	10	100
GTP	31.8	72
ITP	30	100
CTP	0	9
TPP	0	0
UTP	0	3.5
ADP	0	0
PP_i	0	0

It seems to be specific for purine nucleotide triphosphates. It is competitively inhibited by ADP (Fig. 2). The K_m for the reaction with ATP is 0.98 mM.

The ATPase is active in the pH range 6.0–8.5 with optimum activity in the range 7.2–7.8, and is active in the presence of 0.01 % Triton X-100. It is inactivated by 0.1 % Triton X-100 and by 0.1 % sodium dodecyl sulfate.

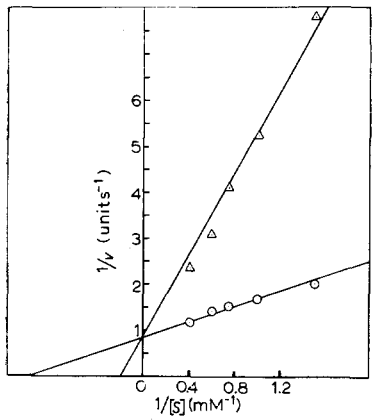

Fig. 2. Competitive inhibition of ATPase by ADP. The soluble ATPase (Stages 5 and 6) was assayed with varying concentrations of ATP as described under METHODS, in the presence of $2.1 \cdot 10^{-4}$ M ADP, and in the absence of ADP. △—△, + ADP; ⊙—⊙, no ADP.

TABLE IV

RECOMBINATION OF SOLUBILIZED ATPase TO CYTOPLASMIC MEMBRANES OF *B. megaterium* KM

Membranes depleted of ATPase and undepleted membranes were incubated at 23° for 30 min with solubilized ATPase. Each tube contained 3.4 mg of membranes, 0.13 mg of solubilized ATPase of specific activity 100 units, 0.01 M $CaCl_2$ and 0.06 M Tris–HCl (pH 7.5) in a total volume of 2 ml. The mixtures and controls were then centrifuged at 18000 × *g* for 10 min to separate the membranes from the supernatants which were poured off. The membranes were resuspended in 0.03 M Tris–HCl (pH 7.5). The different fractions were then assayed for ATPase activity as described in METHODS.

	ATPase activity (*μmoles P_i/10 min at 37°*)	
	Supernatant	*Membranes*
Undepleted membranes	—	3.5
Supernatant	13	—
Undepleted membranes + supernatant	13	3.8
Depleted membranes	—	6.6
Supernatant	13	—
Depleted membranes + supernatant	1.5	10.1

Rebinding of solubilized ATPase to cytoplasmic membranes

Table IV shows that the solubilized ATPase binds back to cytoplasmic membranes which have been depleted of ATPase in the presence of 0.01 M $CaCl_2$, but does not bind back to undepleted membranes under the same conditions. This suggests that the rebinding occurs at sites on the membrane which are specific for the ATPase.

In three separate experiments we found that with the undepleted membranes the total units of activity bound back to the membrane are less than the units of ATPase activity which disappear from the supernatant. The most likely explanation for this is that some of the ATPase is denatured when it is bound back to the membranes under the conditions we use. The results of ISHIDA AND MIZUSHIMA[11] show the same discrepancy.

Purification

DEAE-cellulose was used to purify the solubilized ATPase further. After precipitation with 80 % $(NH_4)_2SO_4$ (refs. 7, 11), we sometimes had difficulty in redissolving all of the precipitate in Tris–HCl buffer even with prolonged dialysis and we did not get increased specific activity of the ATPase (contrast ISHIDA AND MIZUSHIMA[11]). Similarly, protamine sulfate precipitation which substantially increased the specific activity of the ATPase from *B. megaterium* NRLL B929[11] did not increase the specific activity of our ATPase. In our system the batch method used was preferable to the column method using DEAE-cellulose[11], since it resulted in higher specific activity. This may be due to the fact that even when ATP is present to protect the enzyme, the ATPase seems sensitive to inactivation when absorbed on DEAE-cellulose for long

TABLE V

RECOVERY AND SPECIFIC ACTIVITY OF PURIFIED ATPase ISOLATED FROM CYTOPLASMIC MEMBRANES OF *B. megaterium* KM

Supernatants from Stages 5 and 6 were routinely used for further purification of the ATPase. The specific activities of these fractions varied between 40–70 μmoles P_i/mg protein per 10 min at 37°. Ribonuclease (1 μg/ml solution) was added to the supernatants and ATPase activity was precipitated by adding solid $(NH_4)_2SO_4$ to 80% saturation. This precipitate could be stored in the refrigerator for up to 3 weeks without loss of activity. Before use, the precipitate was centrifuged, dissolved in 0.003 M Tris–HCl buffer (pH 7.5) to a protein concentration of approx. 0.5 mg/ml and dialysed against at least two changes of the same buffer for 2 h before use. Any insoluble matter was centrifuged away and discarded. EDTA was then added to a final concentration of 2 mM, and ATP to a final concentration of 1 mM. This solution was added to DEAE-cellulose which had been pre-equilibrated with about 50 ml of buffer (0.02 M Tris–HCl (pH 7.5), 2 mM EDTA, 1 mM ATP). The solution was batch filtered on a Büchner funnel. All the activity remained on the DEAE-cellulose. The DEAE-cellulose was then washed with 20 ml of the same buffer, and 30 ml of buffer containing 0.1 M $(NH_4)_2SO_4$ were passed through the column. The column was washed again with buffer and then 30 ml of buffer containing 0.25 M $(NH_4)_2SO_4$ were passed through. This filtrate contained ATPase of very high specific activity. It was then precipitated with $(NH_4)_2SO_4$ to 80% saturation and stored in the refrigerator for further use.

	Total protein (mg)	*ATPase activity*	
		Total activity (units)	*Specific activity (units/mg protein)*
1. Stages 5 and 6, highly active supernatant released from cytoplasmic membranes with 0.003 M Tris–HCl	31.8	1490	47
2. Not absorbed on DEAE-cellulose equilibrated with 0.02 M Tris–HCl (pH 7.5), 2 mM EDTA, 1 mM ATP	6.2	0	0
3. Eluted from DEAE-cellulose with 0.1 M $(NH_4)_2SO_4$	1.63	354	220
4. Eluted from DEAE-cellulose with 0.25 M $(NH_4)_2SO_4$	0.24	208	870

periods of time, and the batch method has the advantage of greater speed. Table V shows the procedure used and the increase in specific activity with each step. The specific activity of the ATPase from the final step is normally in the range of 450–1000. This range probably reflects variable inactivation of the enzyme during the purification procedures, since the polyacrylamide gel electrophoresis profiles of the different preparations are very similar.

Polyacrylamide gel electrophoresis of the purest fraction using 7.5 % gels and Tris–glycine buffers at pH 8.8 and 8.0 shows a single major protein band with traces of one minor band (Fig. 3). All of the ATPase activity is found in the major band. Sodium dodecyl sulfate gels of the pure fraction show one major band of molecular weight 69000 (Figs. 4 and 5). Fig. 4 shows the purification achieved as measured by the number of bands seen on staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis. We estimate from the gels that the protein is over 90 % pure. Since the active ATPase is eluted in the void volume of a Sephadex G-100 column at pH 7.5,

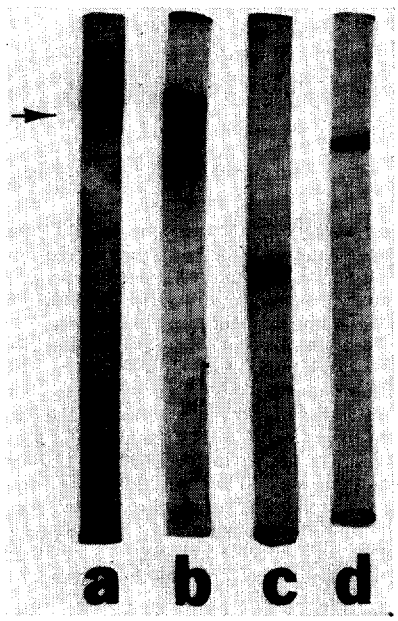

Fig. 3. Polyacrylamide gel electrophoresis of purified ATPase. a,b, 7.5% acrylamide gel, Tris–glycine buffer (pH 8.0). a, gel stained for ATPase as described in METHODS; b, gel stained for protein with Coomassie brilliant blue; c, 7.5% acrylamide gel, Tris–glycine buffer (pH 8.8) stained for protein; d, 10% acrylamide gel, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate (pH 7.0) stained for protein.

its molecular weight is greater 100000. The ATPase subunits appear to be a major band in the sodium dodecyl sulfate polyacrylamide gel electrophoresis profile of whole cytoplasmic membranes (see Fig. 4).

DISCUSSION

The properties of the ATPase from *B. megaterium* KM are similar to those of other membrane bound bacterial ATPases which have been isolated and purified and

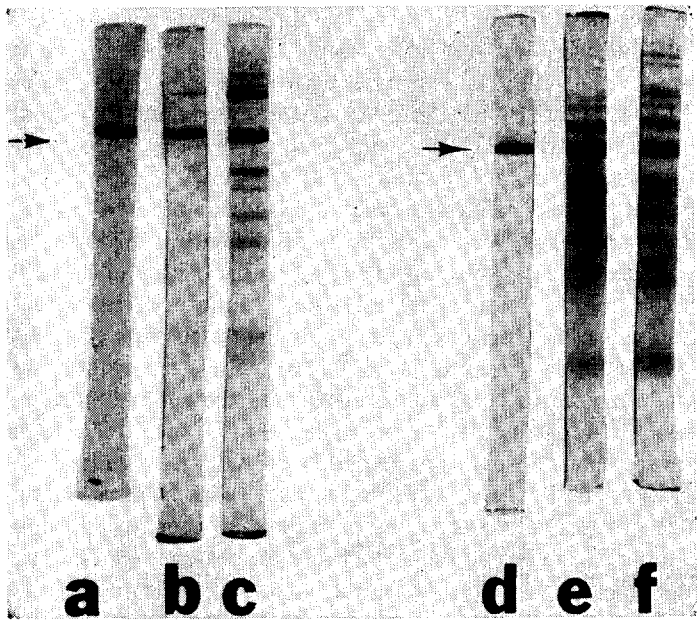

Fig. 4. Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. See METHODS for details of procedure. a,d, purified ATPase; b, fraction eluted from DEAE-cellulose with 0.1 M $(NH_4)_2SO_4$; c, fraction containing high specific activity ATPase obtained by washing cytoplasmic membranes with 0.003 M Tris–HCl, pH 7.5 (Stages 5 and 6); e, whole cytoplasmic membranes of *B. megaterium* KM; f, whole cytoplasmic membranes *plus* purified ATPase. All gels stained with Coomassie brilliant blue. Solvent front run to same distance from origin in all gels.

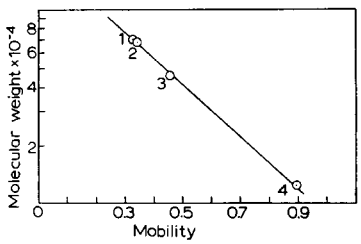

Fig. 5. Molecular weight determination using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Procedure as described under METHODS. 1, purified ATPase; 2, bovine serum albumin; 3, ovalbumin; 4, cytochrome *c*. Molecular weight of ATPase subunit estimated to be 69000. Mobility of standard proteins measured in the same electrophoresis run as that of the purified ATPase to minimize errors due to changes in the running conditions.

also resemble those of the mitochondrial ATPase. However, there are differences, too. We find residual activity in the well-washed membranes as do ABRAMS AND BARON[7] in *S. faecalis* and ISHIDA AND MIZUSHIMA[11] in *B. megaterium* NRLL B929. In contrast, MUÑOZ *et al.*[8] found that the residual ATPase activity in *M. lysodeikticus* was masked and could only be reactivated by treatment with trypsin. The ATPases from *S. faecalis*[5] and mitochondria[3] are activated by Mg^{2+} in preference to Ca^{2+}, whereas those from *M. lysodeikticus*[8] and *B. megaterium* KM are activated further by Ca^{2+} than Mg^{2+} in both the membrane bound and soluble forms. The ATPase from B. *megaterium* NRLL B929 is activated by Ca^{2+} in the soluble but not in the membrane bound form[10], but

we suspect that this is a reflection of the fact that the membranes were prepared differently from our procedure and were not sufficiently depleted of Mg^{2+} used in their preparation before testing. All the ATPases work well with a variety of purine nucleotide triphosphates (ATP, GTP, ITP) but only the mitochondrial ATPase[3] and *B. megaterium* NRLL B929[10] work with UTP as a substrate. ADP is a competitive inhibitor of all the enzymes. ATPases from *S. faecalis* and *M. lysodeikticus* are not cold sensitive unless ADP is present whereas ATPases from mitochondria[3,4], *B. megaterium* NRLL B929[10] and *B. megaterium* KM are all cold sensitive. Mitochondrial ATPase has a molecular weight of 284000 and an $s^{\circ}_{20,w}$ of 11.95 at pH 7.5. It dissociates in the cold into smaller subunits. In the presence of 1 % sodium dodecyl sulfate, it dissociates into a single sedimenting species in the analytical ultracentrifuge with an $s^{\circ}_{20,w}$ 2.65 indicating the presence of subunits[4]. In contrast to ISHIDA AND MIZUSHIMA[10], we found the membrane-bound enzyme to be as cold sensitive as the soluble enzyme. However, it was more resistant to freezing. In the presence of $CaCl_2$, the rebinding of the solubilized enzyme to cytoplasmic enzymes depleted of ATPase parallels the results of ABRAMS AND BARON[6] and ISHIDA AND MIZUSHIMA[11] with *S. faecalis* and *B. megaterium* NRLL B929, respectively, suggesting that divalent ions and, by implication, ionic bonds are important in the binding which occurs between the ATPase and specific sites on the cytoplasmic membrane where it is normally attached.

The highly purified membrane bound ATPase from *B. megaterium* KM has a specific activity at 37° which compares well with a specific activity of approx. 590 ± 60 units at 38° for the membrane-bound ATPase of *S. faecalis*[7]. Specific activities of 294 units and 322 units at 35° were measured for the two peaks of maximal activity obtained after purification of *B. megaterium* NRLL B929 ATPase[11]. When we used the same purification scheme on DEAE-cellulose, we found no trace of a second ATPase peak in our preparations. The purified ATPase from *M. lysodeikticus* has a specific activity of 63 at 37° (ref. 7). Both the high specific activity and the polyacrylamide gel electrophoresis results indicate that we have a highly purified ATPase from the cytoplasmic membrane of *B. megaterium* KM. We are presently working to get rid of the last traces of impurities and to obtain a large enough quantity of the active ATPase to investigate further the chemical and physical properties of the enzyme and its relationship to the whole cytoplasmic membrane.

ACKNOWLEDGMENTS

This work was supported by Grant P 495A from the American Cancer Society, a Grant-in-aid from the New Hampshire Heart Association and by General Research Support Grant FR-05932 from the General Research Support Branch, Division of Research Facilities and Resources, National Institutes of Health.

REFERENCES

1 R. MIRSKY, *Biochemistry*, 8 (1969) 1164.
2 R. MIRSKY, *Arch. Biochem. Biophys.*, 139 (1970) 97.
3 M. E. PULLMAN, H. S. PENEFSKY, A. DATTA AND E. RACKER, *J. Biol. Chem.*, 235 (1960) 3322.
4 H. S. PENEFSKY AND R. C. WARNER, *J. Biol. Chem.*, 240 (1965) 4694.
5 A. ABRAMS, *J. Biol. Chem.*, 240 (1965) 3675.
6 A. ABRAMS AND C. BARON, *Biochemistry*, 7 (1968) 501.
7 A. ABRAMS AND C. BARON, in *Microbial Protoplasts, Spheroplasts and L-Forms*, Williams and Wilkins, Baltimore, 1968, p. 163.

8 E. MUÑOZ, M. R. J. SALTON, M. H. NG AND M. T. SCHOR, *Eur. J. Biochem.*, 7 (1969) 490.
9 T. L. WHITESIDE AND M. R. J. SALTON, *Biochemistry*, 9 (1970) 3034.
10 M. ISHIDA AND S. MIZUSHIMA, *J. Biochem. Tokyo*, 66 (1969) 33.
11 M. ISHIDA AND S. MIZUSHIMA, *J. Biochem. Tokyo*, 66 (1969) 133.
12 E. LAYNE, *Methods Enzymol.*, 3 (1957) 450.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 81 (1929) 629.
15 A. L. SHAPIRO, E. VINUELA AND J. V. MAIZEL, JR., *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.
16 K. WEBER AND M. OSBORN, *J. Biol. Chem.*, 244 (1969) 4406.