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MOLECULAR WEIGHT, AMINO ACID COMPOSITION AND OTHER PROPERTIES OF MEMBRANE-BOUND ATPase FROM *BACILLUS MEGATERIUM* KM

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SUMMARY

The Ca^{2+}-activated ATPase from *Bacillus megaterium* KM has a molecular weight of 379000 as determined by sedimentation equilibrium in the analytical ultracentrifuge and 410000 as determined from sedimentation and diffusion coefficients. These values compare closely with molecular weights estimated for similar ATPases from *Streptococcus faecalis* and mitochondria. On polyacrylamide gel electrophoresis in sodium dodecylsulfate two classes of subunit of molecular weight 68000 and 65000 are seen. They seem to be present in approximately equal proportion. The amino acid analysis gives a minimum molecular weight of 60250 and the amino acid composition is extremely close to that of *S. faecalis*. The enzyme is denatured at 55 °C and insensitive to oligomycin or ruthenium red in either the membrane-bound or soluble forms. The ATPase is estimated to comprise approximately 1% of the total cytoplasmic membrane protein.

INTRODUCTION

In previous papers we have described two methods of purifying a membrane bound Ca^{2+}-activated ATPase from *Bacillus megaterium* KM and some of its properties[1,2]. This paper reports the molecular weight, amino acid analysis and some additional properties of the enzyme.

Several other membrane bound ATPases have been purified and their molecular weight, subunit structure and amino acid composition determined. The Mg^{2+}-activated ATPase from *Streptococcus faecalis* has a molecular weiht of 385000 and an $s_{20,w}$ value of 13.4. It consists of twelve subunits of mol. wt 33000. The subunits are of two distinct types[3,4]. The $s_{20,w}$ value of membrane-bound ATPase from *Micrococcus lysodeikticus* is 14.6 S and that from *B. megaterium* NRLL B929 is 12 S at 21 °C[5,6]. The Mg^{2+}-activated ATPase from the inner membrane of mitochondria has similar properties to the bacterial membrane-bound ATPases and comparable molecular weight and amino acid composition. Subunit weights for rat liver mitochondrial ATPase are reported as 62500, 57000 and 36000 (ref. 7). However, considerable discrepancies exist between the different published molecular weights of the mitochondrial enzyme and its subunits. Earlier studies reported a molecular weight of 284000 for the native en-

zyme with a subunit weight of 26000 [8]. Other studies report a molecular weight of 46000 for the subunits[9].

Our results show that *B. megaterium* KM ATPase has an amino acid composition similar to that of *S. faecalis* ATPase, and also a comparable molecular weight. The molecular weights of the subunits, however, seem to be different, and the enzyme is not sensitive either to oligomycin or ruthenium red.

EXPERIMENTAL SECTION

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 once 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].

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 0.003 M Tris–HCl (pH 7.5). ATPase released in the last two washes was then further purified by centrifugation in a 7–30% glycerol gradient as described previously[2]. In experiments where highest purity ATPase was required we always used enzyme which was run a second time on the glycerol gradient.

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 acitivity 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 as previously described[1]. In experiments where Mg^{2+}-activated ATPase activity was being measured, 2 mM $MgCl_2$ was substituted for $CaCl_2$ in the assay mixture. To test the effect of ruthenium red on the ATPase activity in the presence of both Ca^{2+} and Mg^{2+}, the solution contained 10 mM $CaCl_2$ and 2 mM $MgCl_2$.

Polyacrylamide gel electrophoresis

Polyacrylamide gels containing 0.1% sodium dodecyl sulfate were made as described by Weber and Osborn[12]. Samples were normally heated for 3 min at 100 °C in the presence of 0.1% sodium dodecyl sulfate, 0.1% mercaptoethanol before running on the gels. Approximate molecular weights were estimated using bovine serum albumin, ovalbumin and cytochrome *c* as molecular weight markers. We also used 7.5% and 5% polyacrylamide gels containing 0.015 M Tris and glycine titrated to pH 8.0. 8 M urea was incorporated into some gels, and into the samples run in those gels, and the gels were stained for protein and ATPase activity as described previously[1].

Ultracentrifuge studies

A Beckman Model E analytical ultracentrifuge equipped with both schlieren

and interference optics was used. All runs were made at 20 °C in double-sector synthetic boundary cells. Sedimentation equilibrium measurements were made using the Yphantis[13] meniscus depletion method. The weight average molecular weight was calculated from the slopes of the natural logarithm of the corrected fringe displacement (f) against the square of the radial distance (r) as follows:

$$\text{mol. wt} = \frac{2RT}{(1-\bar{v}\rho)\omega^2}\frac{d\ln f}{dr^2} \quad (1)$$

Where R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the ATPase and ρ is the density of the solvent. Molecular weight was also determined from sedimentation velocity and diffusion experiments using the equation:

$$\text{mol. wt} = \frac{RTs}{D(1-\bar{v}\rho)} \quad (2)$$

where s is the sedimentation constant at a single finite concentration and D is the diffusion constant at the same concentration. Sedimentation velocity runs were made at rotor speeds 42040 rev./min and 39460 rev./min. Diffusion runs were made as described by Chervanka[14] at a speed of 4609 rev./min using a synthetic boundary cell and schlieren optics.

Amino acid analysis

This was performed in a Beckman automatic acid analyzer. The purified protein was dialysed extensively against water and was then lyophilised and hydrolyzed under reduced pressure at 105 °C for periods of 22 h, 48 h and 72 h as described by Spackman *et al.*[15]. Cysteine and cystine were determined after performic acid oxidation of the enzyme using a modification of the method of Hirs[16]. Serine and threonine values were extrapolated to zero-time and 72-h values for proline, isoleucine and valine were used. Tryptophan was estimated using the method of Beavan and Holiday[17].

Ruthenium red, purchased from Sigma Chemical Co. was made up at a concentration of $1.2 \cdot 10^{-3}$ M in 0.1 M Tris–HCl buffer, pH 7.5. To test its effects as an inhibitor it was added at various concentrations to the enzyme assay mixture before the addition of ATP. Oligomycin solution, 1 mg/ml in 50% ethanol, was added to final concentrations in the assay mixture of 10, 20, 40 and 80 μg/ml.

Sodium dithionite and sodium sulfite solutions, both freshly made in 0.1 M Tris–HCl (pH 7.5) were added to the assay mixtures at a final concentration of $1 \cdot 10^{-3}$ M.

RESULTS

Molecular weight of the native enzyme

In the molecular weight studies we used ATPase which had been twice subjected to glycerol gradient centrifugation, with a specific acitivity of 480 units, which is homogeneous by a number of different criteria[2]. Previously we showed that in the analytical ultracentrifuge the native ATPase from the cytoplasmic membrane of *B. megaterium* KM sediments as a single component with an average $s_{20,w}$ value of 13.6. We have now calculated the molecular weight of the native enzyme both by high-

TABLE I

MOLECULAR WEIGHTS OF NATIVE ATPase AND ITS SUBUNITS

Molecular weights of the native ATPase determined in the Beckman Model E analytical ultracentrifuge as described under Methods.

Preparation	*Protein concn (mg/ml)*	*Rotor speed*	*Method*	*Molecular weights*
1. Native enzyme in 0.03 M Tris–HCl (pH 7.5)	3	39 460	Sedimentation velocity and diffusion S 13.6	410 000 ± 5000
	2	42 040	*D* 2.92	
	1.5	4 609		
2. Native enzyme in 0.1 M Tris–HCl (pH 7.5)	1.3	12 590	Yphantis[13] sedimentation equilibrium	397 800
	0.66	13 410		378 000
	0.3	13 410		362 000
Average				379 000 ± 12 000
3. Subunits in 0.1 % sodium dodecyl sulfate, 0.1 % mercaptoethanol, 8 M urea	0.1		Sodium dodecyl sulfate polyacrylamide gel electrophoresis	1. 68 000 2. 65 000

speed sedimentation equilibrium and from the sedimentation and diffusion coefficients as described under Methods. The second method of calculating molecular weight is generally less accurate than sedimentation equilibrium measurements. However, *B. megaterium* KM ATPase is unstable under a variety of conditions[1] and we felt the tendency of the ATPase to precipitate and denature during the longer periods of time required for equilibrium runs introduced inaccuracies and uncertainties which required use of a second method to confirm the results we obtained. Table I shows molecular weights obtained by the two different methods and Fig. 1 a plot of the sedimentation equilibrium data. Pairs of S and *D* values were determined for each concentration

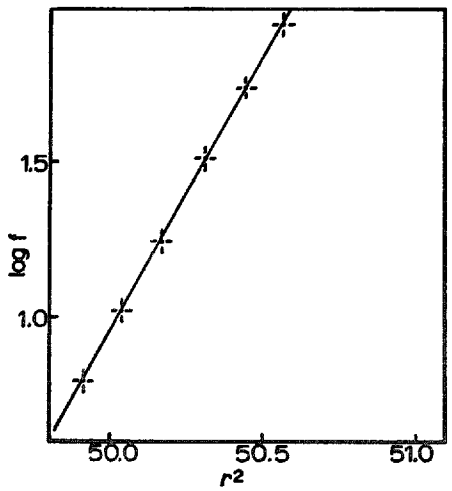

Fig. 1. Typical sedimentation equilibrium data using Yphantis[13] method for ATPase in 0.1 M Tris–HCl (pH 7.5). Plot represents logarithm of fringe displacement against radial distance squared. Protein concentration 0.66 mg/ml, rotor speed 13 410 rev./min, temperature 20 °C.

since these values vary in the same way and thus errors due to concentration are minimized. The partial specific volume of the native enzyme was calculated from the amino acid composition to be 0.730 using the method of Edsall and Cohn[18]. The average molecular weight of the native ATPase is found to be 379000 using the sedimentation equilibrium method and 410000 using sedimentation and diffusion measurements.

Subunits of ATPase

Previous experiments indicated that on polyacrylamide gel electrophoresis of the ATPase in 8 M urea at pH 8.0 two distinct subunits of approximately equal intensity could be seen[2]. A gel scan of the subunits stained with Coomassie blue gives a ratio of 1.1:1 for the relative intensities of the fast and slow bands, though reports indicate that staining with this dye does not always lead to correct quantitative estimation of relative protein concentrations[19]. Unlike Schnebli *et al.*[4] who found a third band in the absence of reducing agents, we found no difference in the band pattern in the presence or absence of mercaptoethanol. Originally we reported that sodium dodecyl sulfate polyacrylamide gel electrophoresis of the ATPase showed only one subunit of mol. wt 69000. However, by repeating these experiments in a slightly different way we now have evidence for the presence of two subunits of slightly differing molecular weights. To eliminate the last traces of impurity, we first ran the twice purified ATPase on 7.5 % Tris–glycine gels at pH 8.0 as described previously[2]. We stained one gel for ATPase activity, then cut out the equivalent portion of two other gels and soaked them overnight in 0.5 ml of 0.003 M Tris–HCl (pH 7.5). We then concentrated the solution to 0.1 ml and added 10 μl of 1 % sodium dodecyl sulfate and mercaptoethanol. The sample was then run on sodium dodecyl sulfate gels in the usual way. Under these conditions two subunits, running close together in the gel, were seen (see Fig. 2).

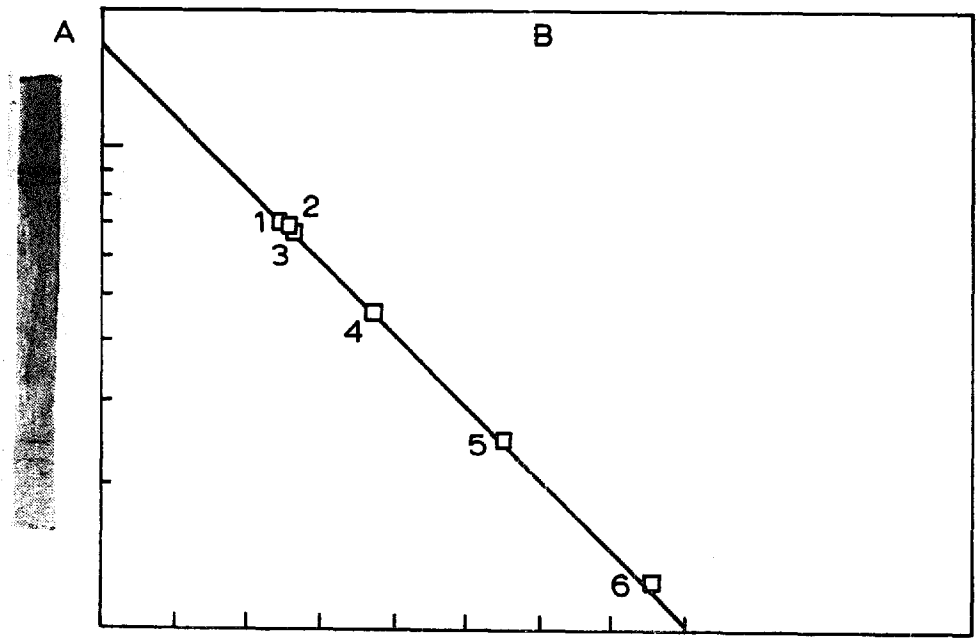

Fig. 2. A. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of ATPase previously run on Tris–glycine polyacrylamide gels. Procedure as described under Results. B. Molecular weight determination using sodium dodecyl sulfate polyacrylamide gel electrophoresis. 1, purified ATPase, Subunit 1; 2, bovine serum albumin; 3, purified ATPase, Subunit 2; 4, ovalbumin; 5, cytochrome *c* dimer; 6, cytochrome *c*. 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.

The molecular weights of the two subunits were estimated to be 68000 and 65000 (Fig. 2) and again the relative intensities of the fast and slow bands stained with Coomassie blue in a ratio of 1.1:1, which corrected for molecular weight yields a molar ratio of 1.17:1.

To investigate further the nature of the ATPase subunits we ran a sample on a Sephadex G-150 column containing 6 M urea and 0.003 M Tris–HCl (pH 7.5). The ATPase was dissolved in 8 M urea prior to application on the column. Unfortunately, the 6 M urea altered the characteristics of the dextran so that it behaved more like G-75 and a bovine serum albumin sample in 8 M urea was eluted in a volume only slightly greater than the column void volume measured with blue dextran. The ATPase sample emerged at almost the same elution volume as the bovine serum albumin, but two peaks could not be distinguished. We also dissolved the ATPase in 6 M guanidine·HCl. On sedimentation velocity of the sample in the analytical ultracentrifuge a single peak was seen, but unlike the profile of the native ATPase it was very broad, perhaps indicating the presence of more than one molecular weight species, so that we felt results obtained by this method would be difficult to interpret.

The amino acid composition of the membrane-bound ATPase from *B. megaterium* KM is given in Table II. Cysteine and cystine are either absent or present in extremely low quantity. No trace of cysteic acid could be detected in the 22 h, 48 h or 72 h hydrolysates and the value obtained on performic acid oxidation is very small. The fact that we find no difference between band patterns of samples run in 8 M urea on Tris–glycine polyacrylamide gels at pH 8.0, both with and without mercaptoethanol also lends support to the conclusion that there are no sulfhydryl groups present.

TABLE II

AMINO ACID COMPOSITION OF *B. megaterium* KM ATPase

Samples of ATPase, twice run on a 7–30% glycerol gradient were dialysed exhaustively against water, and then lyophilised. After the addition of 6 M HCl tubes were evacuated and sealed, then incubated at 105 °C for 22, 48 and 72 h. Some samples were oxidized with performic acid prior to hydrolysis. Tryptophan was estimated spectrophotometrically. For further details see Methods. Values for *S. faecalis* ATPase taken from Schnebli *et al.*[4].

Residue	*Mole percent*	*S. faecalis* (*mole percent*)
Alanine	8.8	8.4
Valine	8.9	6.8
Leucine	9.3	9.3
Isoleucine	6.3	6.2
Proline	4.3	3.9
Phenylalanine	3.3	3.1
Methionine	2.0	2.3
Glycine	9.0	8.7
Serine	5.6	6.3
Threonine	5.9	6.7
Half-cystine	0.1	0.325
Tyrosine	2.7	3.3
Aspartate	8.5	10.0
Glutamate	13.4	13.0
Arginine	5.6	4.5
Lysine	5.1	6.1
Histidine	1.6	1.7
Tryptophan	0.4	—

Schnebli *et al.*[4] found different band patterns with and without the reducing agent dithiothreitol. The minimum molecular weight of *B. megaterium* KM ATPase, calculated from the amino acid analysis is 6250, so that it is possible that there is one sulfhydryl group for every two subunits, assuming an average weight of 66000.

We decided to test the sensitivity of the ATPase to both oligomycin, which inhibits the mitochondrial membrane-bound ATPase[20] and to ruthenium red which is reported to be a specific inhibitor of a variety of Ca^{2+}-activated ATPases[21]. Table III shows that in both the soluble and membrane-bound forms, *B. megaterium* KM ATPase is unaffected by both of these compounds. Soluble Mg^{2+}-ATPase activity is sti-

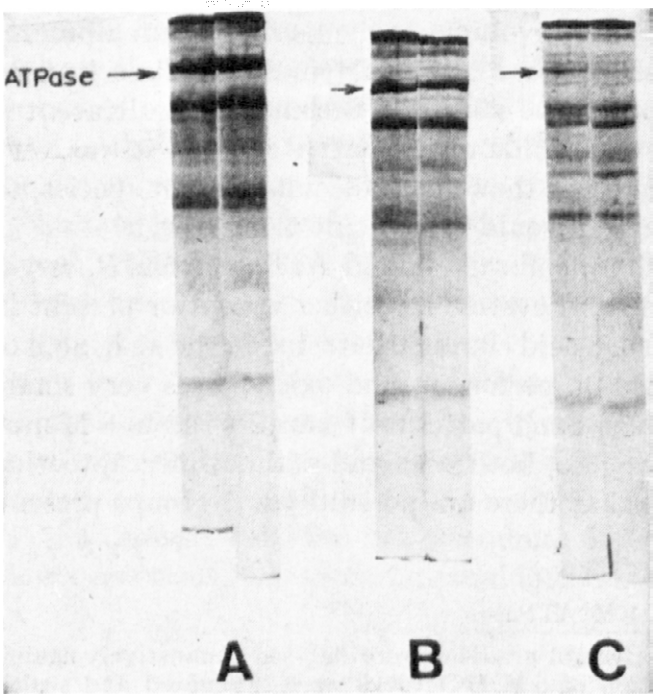

Fig. 3. Heat treatment of ATPase. Polyacrylamide gel electrophoresis on Tris–glycine gels (pH 8.0). High activity ATPase obtained by washing cytoplasmic membranes with 0.003 M Tris–HCl (pH 7.5). A, supernatant after heat treatment at 50 °C for 5 and 10 min, respectively; B, supernatant after heat treatment at 55 °C for 5 and 10 min, respectively; C, supernatant after heat treatment at 60 °C for 5 and 10 min, respectively.

TABLE III

SENSITIVITY OF ATPase TO VARIOUS COMPOUNDS

Compounds were added to assay mixture just before addition of ATP. Assay conditions were otherwise as described under Methods. Compounds were tested over a range of ATPase concentrations.

Compound	*Concentration*	*ATPase activity (% of control)*					
		Membrane bound			*Soluble*		
		Ca^{2+}	Mg^{2+}	$Ca^{2+} + Mg^{2+}$	Ca^{2+}	Mg^{2+}	$Ca^{2+} + Mg^{2+}$
Oligomycin	10–40 μg/ml	100	—	—	100	—	—
Ruthenium red	$1 \cdot 10^{-6}$–$1 \cdot 10^{-5}$ M	100	95	101	105	100	97
Dithionite	$1 \cdot 10^{-3}$ M	—	—	—	107	152	—
Na_2SO_3	$1 \cdot 10^{-3}$ M	—	—	—	96	99	—
Ethanol	2 %	69	—	—	95	100	—

mulated by dithionite, and membrane-bound Ca^{2+}-ATPase is partially inactivated by 2% ethanol.

The ATPase is stable up to 50°C, but is 18% inactivated when heated for 5 min. at 55°C, 39% inactivated after 10 min at 55°C, 78% inactivated when heated for 5 min at 60°C, and 90% inactivated after 10 min at 60°C. Fig. 3 shows the progressive disappearance of the ATPase from solution at 55 and 60°C. The precipitate contains no detectable ATPase acitivity.

DISCUSSION

The molecular weight of *B. megaterium* KM membrane ATPase is similar to that reported for ATPases of similar type such as *S. faecalis* ATPase[4] and mitochondrial ATPase[7]. The differences between the molecular weight of *B. megaterium* KM ATPases determined by sedimentation velocity and diffusion coefficients represent a difference of 7.5%, an error well within the accuracy of the methods used. Assuming subunit molecular weights of 68000 and 65000 and equal proportions of each subunit we estimate that there are three subunits in each molecular weight class with a total of six subunits per molecule. This would give a total molecular weight for the native enzyme of 399000, a result in good agreement with the molecular weight determined by other means. The subunit molecular weights obtained for *B. megaterium* KM ATPase are fairly similar to the larger subunit molecular weights of 62500 and 57000 determined for rat liver mitochondrial ATPase, although in the latter enzyme, a smaller subunit of mol. wt 36000 also seems to be present. In sodium dodecyl sulfate gels run using ATPase cut out and eluted from Tris–glycine gels we did not see this band in *B. megaterium* ATPase. The subunit weight of 33000 reported for *S. faecalis* ATPase was determined by sedimentation equilibrium experiments of the enzyme dissolved in 6 M guanidine·HCl. Under these conditions we could not obtain a sharply defined peak in sedimentation velocity experiments in the ultracentrifuge using schlieren optics, so no direct comparison is possible. It should be emphasized that it is not possible to tell whether the subunits in the same molecular weight class, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, are identical or not.

The amino acid composition of *B. megaterium* KM ATPase is very similar to that reported for *S. faecalis* ATPase, with the important difference that the *B. megaterium* KM ATPase seems to contain either few or no sulfhydryl groups. Considering the close similarities in the primary structure of the two enzymes it is interesting that *S. faecalis* ATPase is relatively insensitive to cold inactivation and is activated by Mg^{2+}, whereas *B. megaterium* KM ATPase is cold sensitive and activated by Ca^{2+} in preference to Mg^{2+} (ref. 1). The similarities between the amino acid compositions and properties of ATPases from *S. faecalis*, rat liver mitochondrial ATPase, beef heart mitochondrial ATPase and spinach chloroplast ATPase have been commented on elsewhere[7], and *B. megaterium* KM ATPase obviously forms part of the same group. The insensitivity to oligomycin is found in other bacterial membrane ATPases. Ruthenium red is reported to be highly specific for Ca^{2+}-ATPase activity as opposed to Mg^{2+}-, Na^{+}- and K^{+}-ATPase activity[21], but it does not inhibit *B. megaterium* KM Ca^{2+}-activated ATPase over the concentration range in which it inhibits erythrocyte membrane Ca^{2+}-ATPase. Nor does it inhibit Mg^{2+}-activated ATPase, or ATPase determined in the presence of both

Ca^{2+} and Mg^{2+}. Its use as a general tool for studying the properties of Ca^{2+}-ATPases may therefore be limited to specific classes of Ca^{2+}-ATPases.

Cytoplasmic membranes undepleted of ATPase have a specific acitivity ranging from 4–6 units/mg membrane protein. Compared with the value for the purified ATPase of between 400–600 units[1,2] this would suggest that ATPase forms approximately 1 % of the total membrane protein which compares reasonably with a value of about 2 % for the ATPase from *S. faecalis* membrane[4].

B. megaterium KM ATPase seems to be part of a general class of ATPases with similar chemical and physical properties which are distributed through the bacterial, animal and plant kingdoms. Although it is known that mitochondrial ATPase is involved in oxidative phosphorylation, spinach chloroplast ATPase is involved in photosynthetic phosphorylation, and *S. faecalis* ATPase particpates in the ATP-driven translocation of some amino acids[7], the detailed way in which any of these ATPases function when coupled with other enzyme systems *in vivo* has yet to be worked out.

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