Biochimica et Biophysica Acta, 777 (1984) 151–154
Elsevier

BBA Report

BBA 70183

PROPERTIES OF THE MEMBRANE-BOUND Mg^{2+}-ATPase ISOLATED FROM *ACHOLEPLASMA LAIDLAWII*

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(Received April 2nd, 1984)

Key words: Mg^{2+}-ATPase; Subunit composition; Molecular weight; Amino acid composition; (A. laidlawii)

Mg^{2+}-ATPase has been isolated to homogeneity from *Acholeplasma laidlawii* AIHO89 membrane by extraction with 2% Triton X-100 followed by polyacrylamide gradient gel electrophoresis. The enzyme with its activity retained appeared as a single band in the gradient gel electrophoresis and consisted of five subunits as shown by sodium dodecyl sulfate electrophoresis. The molecular weights of the subunits were found to be 66000, 47000, 34000, 26000, and 11000, respectively. Slight inhibitory effects of oligomycin and DCCD on the Mg^{2+}-ATPase activity were shown. KSCN partially inhibited the enzyme at concentration of 0.1 M. Ouabain had no inhibitory effect on the enzyme at all. High contents of acidic amino acids and hydrophobic amino acids were found in the Mg^{2+}-ATPase.

The Mg^{2+}-ATPase has been found to be tightly associated with the cell membrane of every mycoplasma and localized on the inner cytoplasmic face of the cell membrane. It is an integral protein [1] and depends on membrane lipids for its activity [2]. The Mg^{2+}-ATPase could not be released by the method employed for the solubilization of peripheral membrane proteins. Due to the high sensitivity of *Acholeplasma laidlawii* Mg^{2+}-ATPase to ionic detergents, most of the earlier attempts to purify the enzyme with ionic detergents failed. Non-ionic detergents, however, are often used in solubilization of *A. laidlawii* membrane, and the Mg^{2+}-ATPase can be found in the supernatant [3,4]. Mg^{2+}-ATPase in *A. laidlawii* membrane fragments and supernatant solubilized by detergents has been studied (see Refs. 5–7). But we still know very little about its molecular properties. Very recently, the purification and characterization of the $(Na^+ + Mg^{2+})$-ATPase of *A. laidlawii* B has been reported [8].

Our previous work showed that 50–60% of Mg^{2+}-ATPase activity in *Mycoplasma galliseptium* S_6 strain membrane was recovered in supernatant extracted by 2% Triton X-100 [9]. In the present paper, a method for isolation and detection of the Mg^{2+}-ATPase in *A. laidlawii* AIHO89 by polyacrylamide gradient gel electrophoresis is described and the enzyme is partially characterized.

A. laidlawii (strain AIHO89) was cultivated at 37 °C for 20 h in a medium containing 1000 ml pig heart infusion, 20 g tryptone, 10 g glucose, 3.7 g Tris, 5 g NaCl, 100 ml pig serum and 400000 units of penicillin. The cells were harvested by centrifugation at $9000 \times g$ for 30 min and washed twice with a buffer (0.15 M NaCl/0.05 M Tris/0.01 M β-mercaptoethanol, adjusted to pH 7.4 with HCl). The cellular pellets were lysed in redistilled water. The membranes were collected by centrifugation at $48000 \times g$, followed by two washes with 1 : 20 diluted buffer. Finally the washed membranes were suspended in the same buffer and kept at −40 °C until use. The protein concentration was determined by the method of Lowry et al. [10]. The

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membrane suspension (4 mg/ml) was treated with 2% Triton X-100 at 37 °C for 30 min. Approx. 0.1 ml of the supernatant obtained by centrifugation at 25 000 × *g* for 30 min at 4 °C was layered onto the polyacrylamide gradient gel. After electrophoresis, the gels were removed from the tubes and incubated for 10–20 min at 37 °C in Mg^{2+}-ATPase or *p*-nitrophenylphosphatase reaction medium [11] containing 0.032% lead acetate. The band staining for Mg^{2+}-ATPase and *p*-nitrophenylphosphatase activity on the polyacrylamide gradient gel can be clearly observed (Fig. 1). By comparing the bands with those stained by Coomassie brilliant blue we found that the ATPase and *p*-nitrophenylphosphatase activities appeared in a single band at the position corresponding to the protein band I and II, respectively (Fig. 1). The Mg^{2+}-ATPase protein comprised about one-tenth of the total protein content in the supernatant. In our experiment, 5–10 min incubation at 37 °C was enough for detection of the enzyme activity band when freshly prepared membranes were used.

In another experiment we changed two key factors in the enzyme reaction medium: Mg^{2+} and substrate ATP. The absence of Mg^{2+} or ATP from the incubation medium resulted in the loss of the Mg^{2+}-ATPase activity staining band. The enzyme activity can only be detected when both Mg^{2+} and ATP were present. This provides strong evidence that the white band on the gel was caused by the Mg^{2+}-ATPase in *A. laidlawii* rather than by other kinds of ATPase.

The Mg^{2+}-ATPase in mycoplasma membrane belongs to the group of integral membrane enzymes dependent on lipids for their activity. Most available techniques designed for use with soluble proteins are not easily adapted to the hydrophobic membrane protein. It is necessary to find an adequate assay for its presence and a suitable method for the separation and purification of the Mg^{2+}-ATPase. Attempts to separate the enzyme from *A. laidlawii* membranes solubilized by non-ionic detergents have failed so far [6,12]. The main difficulty lies in the formation of lipid-protein-detergent micellar complexes and reaggregation of the proteins and lipids with dilution or removal of the detergent during ultrafiltration [12]. We have now been able to isolate and identify the Mg^{2+}-ATPase in *A. laidlawii* membrane solubilized by Triton X-100 in an active form by polyacrylamide gradient gel electrophoresis and the ATPase activity staining method. There were no other proteins in the vicinity of the enzyme activity band.

The question whether the ATPase and *p*-nitrophenylphosphatase activities of *A. laidlawii* membrane are the expression of a single enzyme

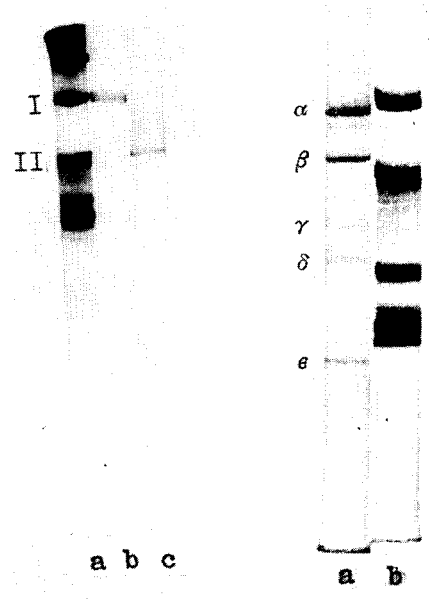

Fig. 1. The isolation and detection of the Mg^{2+}-ATPase and *p*-nitrophenylphosphatase in *A. laidlawii* by 3.5–8% polyacrylamide gradient gel electrophoresis at 4 °C, 100 V, 5 h. (a) The protein bands were stained by Coomassie brilliant blue R-250 (0.25%) in 50% methanol and acetic acid (9:1, v/v) for 10 h and destained in several changes of methanol/acetic acid/water (10:3:27, v/v). (b) The Mg^{2+}-ATPase activity staining band. (c) The *p*-nitrophenylphosphatase activity staining band. Both (a) and (b) gels were incubated for 10 min at 37 °C in Mg^{2+}-ATPase and *p*-nitrophenylphosphatase reaction medium containing 0.032% lead acetate.

Fig. 2. Polyacrylamide gel electrophoresis of the isolated Mg^{2+}-ATPase in sodium dodecyl sulfate. The stacking and separating gel composition were T = 3.5%, C = 3.3% and T = 10%, C = 3.3%, respectively. 0.2 M glycine-Tris solution (pH 8.3) containing 0.1% SDS was used as electrode solution. Electrophoresis was carried out at 4 mA/tube at 4 °C for 4 h. The gel staining and destaining procedures were the same as in Fig. 1. (a) The bands were called α, β, γ, δ, ε, in order of decreasing molecular weight. (b) The standard molecular weight proteins: bovine serum albumin 67000, ovalbumin 43000, Trypsin 23300 and RNAase 13700.

can now be answered negatively. In this paper, direct visual evidence has been provided. Two entirely different enzyme activity bands in different locations on the gel can be clearly observed when different substrates, viz. ATP and *p*-nitrophenylphosphate, are added to the enzyme reaction medium (Fig. 1). Ne'enman et al. [12] suggested that the non-ionic detergents do not seem to disaggregate the membrane completely. Under our experimental conditions, the membrane fragments cannot enter the gel and no enzyme activity was found at the top of the gel, which can be strongly stained with Coomassie brilliant blue. The results tend to show that the micellar complex of the Mg^{2+}-ATPase detected on the gel was homogeneous in size. In order to protect membrane proteins from reaggregation, Triton X-100 has to be included throughout the experimental procedure.

When the supernatant obtained by non-ionic detergent was separated by Sephadex G-200 column chromatography, the enzyme activity appeared in the void volume of the column [12]. In other words, the molecular weight of the enzyme was greater than 500000. Using 4–20% polyacrylamide gradient electrophoresis (4°C, 150 V, 30 h, standard molecular weight proteins: thyroglobulin, ferritin, catalase, lactate dehydrogenase and bovine serum albumin), we estimate the molecular weight of the Mg^{2+}-ATPase to be about 800000. It has been reported that the molecular weights of mycoplasma membrane proteins assessed by SDS-polyacrylamide gel electrophoresis are in the range from 15000 to 200000 [13]. The molecular weight of the H^+-ATPase complex of pig heart mitochondrial membrane is about 460000 [14] and the soluble Mg^{2+}-ATPase from erythrocyte membrane is a hexamer of 600000 comprised of six subunits of 100000 [15]. Since the Mg^{2+}-ATPase in *A. laidlawii* has not been purified, the actual molecular weight of the enzyme cannot yet be ascertained.

Upon re-electrophoresis in a gel containing SDS, the Mg^{2+}-ATPase-active band is resolved into five polypeptides. The apparent molecular weights of the two major bands α and β were 66000 and 48000, and of the minor bands, γ, δ and ϵ, 34000, 26000 and 11000, respectively (Fig. 2). The pattern was very similar to that of *A. laidlawii* B ($Na^+ + Mg^{2+}$)-ATPase in the Weber and Osborn system [8], but a slight difference in molecular weight of each band can be noticed. It was demonstrated that the Mg^{2+}-ATPase of *A. laidlawii* membrane resembles that of F-F_0 type ATPase in consisting of five subunits.

Using a variety of inhibitors known to affect the mitochondrial H^+-ATPase complex and ($Na^+ + K^+$)-ATPase of eukaryotic plasma membranes, we tried to probe the inhibitory effects on the Mg^{2+}-ATPase activity of *A. laidlawii* by the method of activity staining on the gels. The inhibitory effect was shown by the time required for developing the band and the intensity of the bands. No inhibitory effects were observed. Probably, the sensitivity of the staining method was so high that the remaining enzyme activity was sufficient to develop the white bands on the gels. The native membrane Mg^{2+}-ATPase was found to be entirely insensitive to ouabain and only slightly inhibited by DCCD and oligomycin at a higher concentra-

TABLE I

AMINO ACID ANALYSIS OF THE ISOLATED Mg^{2+}-ATPase IN *A. LAIDLAWII*

The Mg^{2+}-ATPase activity staining band was cut out from the gels and extracted by redistilled water overnight. To the filtrate was added an equal volume of 12 M HCl, and the mixture was hydrolyzed at 110°C for 24 h. The amino acid composition was determined with a Hitachi 835-50 automatic amino acid analyzer. The corrections used for destruction of amino acid during hydrolysis are 5% for threonine and tyrosine and 10% for serine [21].

Amino acid	mol%
Aspartic acid	7.7
Threonine	4.5
Serine	9.3
Glutamic acid	9.2
Glycine	14.3
Alanine	6.6
Valine	5.2
Methionine	1.9
Isoleucine	4.8
Leucine	8.0
Tyrosine	1.9
Phenylalanine	13.0
Lysine	4.8
Histidine	1.0
Arginine	3.4
Proline	4.0

tions than generally used for the H^+-ATPase complex. They inhibited by 13% at $1 \cdot 10^{-3}$ M and by 30% at 8 μg/ml of the Mg^{2+}-ATPase activity, respectively. KSCN exhibited a greater inhibition (60%) at a concentration of 0.1 M. These results indicated that the enzyme differs from F_1-F_0-type ATPase in being much more resistant to inhibitors such as DCCD and oligomycin. The enzyme differs from $(Na^+ + K^+)$-ATPase of plasma membrane, too. Table I shows the amino acid composition of the Mg^{2+}-ATPase. The content of acidic amino acids was about 17% of the total amino acid content. The ratio of acidic to basic amino acids was 2 : 1. A high content of hydrophobic amino acids (74%) was noticed. The amino acid composition of membrane proteins in *A. laidlawii* has been reported [16,17]. The majority of *A. laidlawii* proteins were acidic proteins having isoelectric points of 4.5–7.5 as revealed by isoelectric focusing [18]. Our results indicate the content of acidic amino acids in the Mg^{2+}-ATPase to be similar to that of native membranes [17], and rather high. The content of amino acids with hydrophobic side-chains in the Mg^{2+}-ATPase from *A. laidlawii* is higher than that of the soluble Mg^{2+}-ATPase from erythrocyte membrane [19] and the water-soluble F_1 subunit of H^+-ATPase complex [20]. Glycine and phenylalanine in the enzyme are much higher than that in the native membrane [17]. It is possible that these nonpolar amino acids may contribute to hydrophobic interaction with membrane lipids.

In order to investigate the native function of the Mg^{2+}-ATPase by means of reconstitution experiments, it is necessary to remove the detergents from the micellar system. The non-ionic detergents usually possess the undesirable property of having a low critical micelle concentration. Gel filtration chromatography and density gradient centrifugation are not suitable for this system. It is apparent that a great deal of experimental work remains to be done. Further studies are in progress.

We thank Yi-wen Jian, Kang-tao Ma and Baozhen He for assistance with some of the experiments. We also wish to thank professor Rong Liu and Su-min Wang for commenting and assistance in correcting the English version of this manuscript.

References

1 Ne'eman, Z. and Razin, S. (1975) Biochim. Biophys. Acta 375, 54–68
2 Bevers, E.M., Snoeh, G.T., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 346–356
3 Ne'eman, Z., Kahane, I. and Razin, S. (1971) Biochim. Biophys. Acta 249, 169–176
4 Johansson, K.E. and Hjertén, S. (1975) J. Biol. Chem. 250, 2463–2469
5 Rodwell, A.W., Razin, S., Rottem, S. and Argaman, M. (1967) Arch. Biochem. Biophys. 122, 621–628
6 Pollack, J.D., Razin, S. and Cleverdon, R.C. (1965) J. Bacteriol. 90, 617–622
7 Rottem, S. and Razin, S. (1966) J. Bacteriol. 92, 714–722
8 Lewis, R.N.A.H. and McElhaney, R.N. (1983) Biochim. Biophys. Acta 735, 113–122
9 Chen, J.-W., Jiang, Y.-W. and Hwang, F. (1981) Progress Biochem. Biophys. (Chinese) 5, 35–39
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
11 De Kruyff, B., Van Dijck, P.W.M., Goldbach, R.W., Demel, R.A. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 330, 269–282
12 Ne'eman, Z., Kahane, I., Kovartovsky, J. and Razin, S. (1972) Biochim. Biophys. Acta 266, 255–268
13 Hjertén, S. and Johansson, K.E. (1972) Biochim. Biophys. Acta 288, 312–325
14 Pedersen, P.L. (1975) J. Bioenerg. 6, 243–275
15 White, M.D. and Ralston, G.B. (1976) Biochim. Biophys. Acta 436, 567–576
16 Engelman, D.M. and Morowitz, H.J. (1968) Biochim. Biophys. Acta 150, 376–384
17 Morowitz, H.J. and Terry, T.M. (1969) Biochim. Biophys. Acta 183, 276–294
18 Chen, J.-W., Sun, Q. and Hwang, F. (1982) Progress Biochem. Biophys. (Chinese) 5, 40–43
19 Hjertén, S. and Pan, H.-Z. (1983) Biochim. Biophys. Acta 728, 281–288
20 Lin, Z.-H., Wu, E., Sun, S., Xu, G.-Z. and Zhang, K. (1981) Progress Biochem. Biophys. (Chinese) 5, 53–56
21 Moore, S. and Stain, W.H. (1963) Methods Enzymol. 6, 819