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Membrane-associated ATPase activity from *Micrococcus lysodeikticus*

Membrane-bound ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity has been found in some Gram-positive bacteria[1–4]. MITCHELL[5] demonstrated ATPase activity associated with membranes of *Micrococcus lysodeikticus*. ISHIKAWA AND LEHNINGER[6] studying oxidative phosphorylation in this bacterium described a membrane-associated ATPase activity which was released as soluble protein by osmotic shock in the cold. More recently, ISHIKAWA[7] reported the identity of a Ca^{2+}-activated ATPase and a coupling factor associated with the membrane *M. lysodeikticus*. The activity of this Ca^{2+}-ATPase was very low and was increased by trypsin treatment. With the exception of these findings, very little is known about the characteristics of this enzyme and any functional relationship between these characteristics and the bacterial membrane. This preliminary report describes some results obtained in our bacterial membrane studies of a membrane-bound ATPase in *M. lysodeikticus*, its solubilization in a similar way to the membrane-bound ATPase from *Streptococcus faecalis*[4], and several of its properties.

M. lysodeikticus NCTC 2665 was grown and harvested as described previously[8]. Membranes were obtained from protoplasts by osmotic shock. The cells (20–30 mg dry weight/ml) were resuspended in 0.03 M Tris–HCl buffer at pH 7.5 and made 0.8 M with respect to sucrose and 0.05 M for $CaCl_2$ or $MgCl_2$. Cells were held for 30 min at room temperature (20–25°) under these hypertonic conditions. Lysozyme (200 μg/ml) was then added and after 30 min-incubation at room temperature, the protoplasts so formed were harvested by centrifugation at 0° (30 min at 30000 $\times$ g in a Sorvall RC-2 centrifuge). The protoplasts were then burst in 0.03 M Tris–HCl buffer at pH 7.5 in the presence of deoxyribonuclease (20 μg/ml). Membranes were sedimented by centrifugation at 0° (30-min at 30000 $\times$ g) and washed several times with the same buffer. All supernatants were recovered and assayed for protein content[9] and ATPase activity. The activity was measured by the liberation of inorganic phosphate (P_i) in a test system that contained: ATP 8 μmoles, $CaCl_2$ or $MgCl_2$ 4 μmoles, Tris–HCl buffer 90 μmoles and 10–50 μg of protein, in a final volume of 1 ml at pH 7.5. After 10 min of incubation at 37° the reaction was stopped with 0.1 ml of 20% trichloroacetic acid and 0.5 ml of the supernatant solution was removed for P_i determination[10].

Although the supernatant from the first wash containing the soluble cytoplasmic proteins showed no ATPase activity, the supernatants from the third, fourth and fifth washings had a high specific activity for this enzyme. The pooled active supernatants prepared in 0.03 M Tris–HCl buffer at pH 7.5 were stable at 4° for several weeks. The ATPase activity showed a clear requirement for the divalent cations Ca^{2+} or Mg^{2+}. Ca^{2+} was the better activator and its presence resulted in a specific activity of 4 μmoles of P_i/min per mg of protein. As an activator, Mg^{2+} was less efficient and gave a specific activity of 1 μmole P_i/min per mg of protein. It was significant that Mg^{2+} was an inhibitor of the Ca^{2+}-ATPase activity and gave 50% inhibition when the Mg^{2+} concentration was one-tenth that of the Ca^{2+}. 2,4-Dinitrophenol, Na^+ and K^+ had no influence on the Ca^{2+}-activated ATPase.

The enzyme did not hydrolyse ADP or *p*-nitrophenylphosphate. Moreover, ADP was an inhibitor of the Ca^{2+}-activated ATPase (70% inhibition with $4 \cdot 10^{-3}$ M

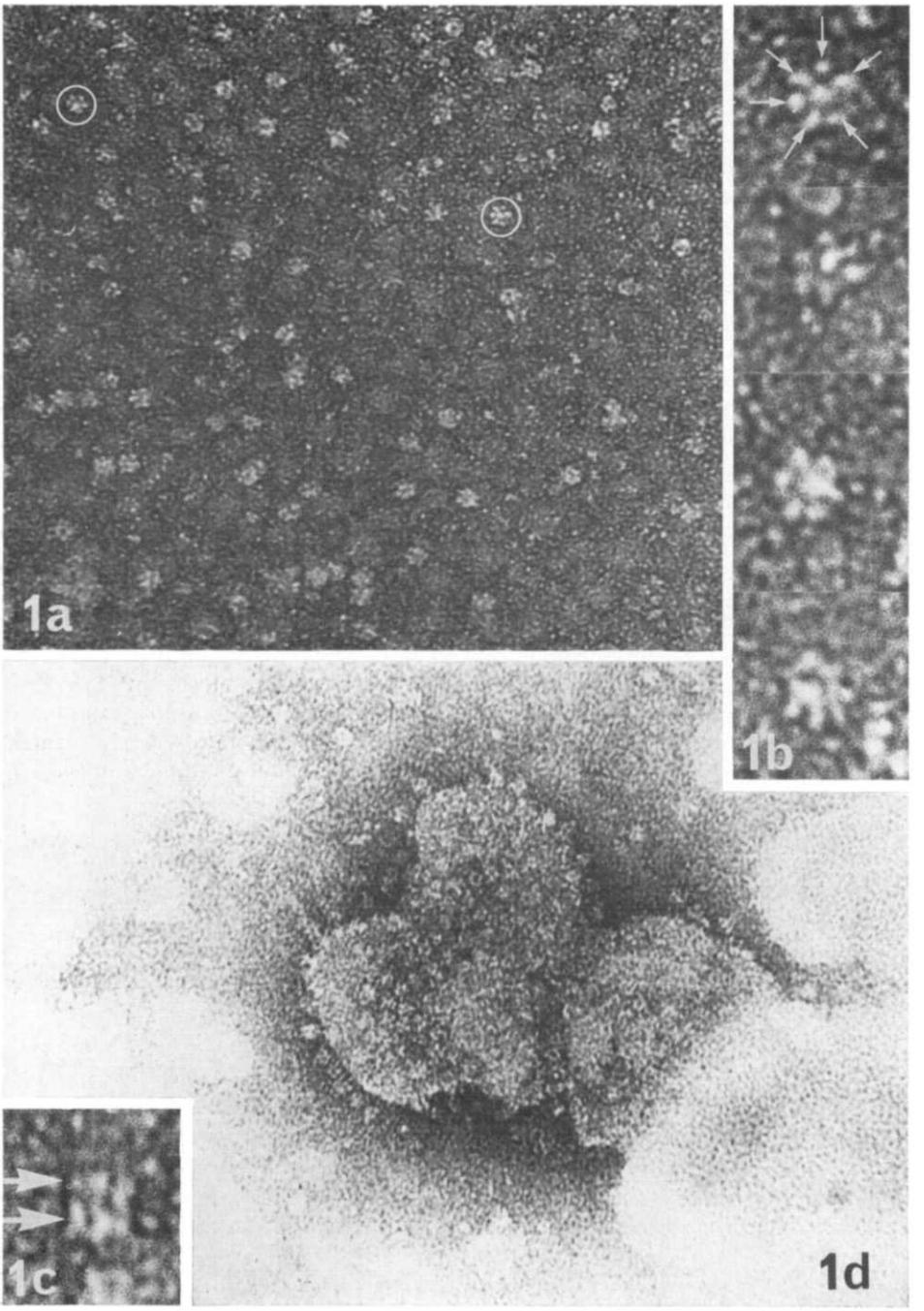

Fig. 1. Negatively stained ATPase preparation. a. Active enzyme preparation showing spherical particles of approx. 100 Å diameter (circles), × 300 000. b. Selected particles showing a central subunit surrounded by 6 additional subunits (arrows), × 1000 000. c. Selected particles which appear to consist of 2 apposed discs of subunits (arrows), × 1000 000. d. Membrane fragment showing associated particles, × 300 000.

ADP). Of the usual inhibitors of ATPase (*p*-chloromercuribenzoate, *N*-ethylmaleimide, ouabain, cyanide, sodium azide), only azide was effective with 60 % inhibition at $1 \cdot 10^{-4}$ M azide. The ATPase activity was destroyed by incubation with trypsin, but was not affected by deoxyribonuclease treatment. With respect to these properties and the high specific activity, our enzyme differs from the preparation of ISHIKAWA[7]. This may be the result of differences in the methods of preparation of the enzymes or may indicate that they are different proteins.

The active enzyme preparation was examined in the electron microscope using negative staining with 1.0 % ammonium molybdate in 2.0 % ammonium acetate buffer (pH 7.2). The appearance of the enzyme is seen in Fig. 1. The active enzyme fraction (Fig. 1a) appears to consist mainly of roughly spherical particles of approx. 100 Å diameter. Subunits can be seen in many of the particles. Fig. 1b shows particles at a higher magnification, two of which reveal the presence of a central subunit encircled by 6 additional units. Certain particles have a rectangular profile which may indicate that the particles are in fact disc shaped and can associate, at least in pairs, to form short cylinders (Fig. 1c). Particles similar to those seen in the purified enzyme preparations were often found associated with membrane fragments (Fig. 1d). Particles of similar dimensions have been seen associated with membrane fragments in ATPase-rich fractions obtained from mitochondria[11,12]. ABRAM[13] and BIRYUZOVA *et al.*[14] have described stalked particles of about the same size associated with bacterial membranes. The association of ATPase activity with these particles suggests a basic type of structural organization common to mitochondrial and bacterial membrane systems.

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