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PURIFICATION AND CHARACTERIZATION OF THE MEMBRANE ($Na^+ + Mg^{2+}$)-ATPase FROM *ACHOLEPLASMA LAIDLAWII* B

RUTHVEN N.A.H. LEWIS and RONALD N. McELHANEY

Department of Biochemistry, University of Alberta, Medical Sciences Building, Edmonton, Alberta, T6G 2H7 (Canada)

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The membrane ($Na^+ + Mg^{2+}$)-ATPase of *Acholeplasma laidlawii* B has been solubilized with a Brij-58/sodium deoxycholate mixture and purified by a combination of gel filtration and ion-exchange chromatography. The purified, partially delipidated ATPase has a specific activity of 195 μmol P_i/mg protein per h, which could be enhanced by 25% upon the addition of exogenous phospholipids. The kinetic properties of the purified enzyme are similar to those of the native membrane-bound enzyme, suggesting that it has not been substantially altered during the purification procedure. The enzyme is an assembly of five polypeptide species and its kinetic properties suggest that it is dissimilar to other known ATPases.

Introduction

The organism *Acholeplasma laidlawii* B has a single membrane, its plasma membrane, with which its ($Na^+ + Mg^{2+}$)-ATPase activity is associated [1]. This enzymic activity has been characterized in membrane fragments with respect to its kinetic properties [2,3] and phospholipid requirements [4]. Manipulation of the fatty acid composition of the plasma membrane of this organism [5] has been very useful in studies on the lipid phase-state dependence of its ATPase activity [3,6]. However, the inability to make significant alterations in the phospho- and glycolipid composition of this membrane has limited studies on the lipid headgroup dependence of this enzyme, and has necessitated the purification of this enzyme for reconstitution into defined lipid systems. Most attempts at the purification of this ATPase have met with limited success, primarily as a result of problems associated with the solubilization of integral membrane proteins from this organism while retaining the biological activities of such proteins [7]. A procedure for the purification of this enzyme has been recently reported [8], but the structural and kinetic porperties of that purified preparation were not described. We report here a procedure for the solubilization of *A. laidlawii* B ($Na^+ + Mg^{2+}$)-ATPase and for the purification of the enzyme in a soluble, active form complexed with lipid and detergent. The characterization of the purified ATPase suggests that this enzyme may be different from any of the purified ATPases previously studied.

Materials

The organism used for this work is *A. laidlawii* B originally obtained from Wellcome Research Laboratories. Medium components were obtained from Difco Laboratories. Sigma Chemical Co.

Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DMSO, dimethylsulfoxide; DCCD, *N,N,N',N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; P_i, inorganic phosphate; DTSP, dithiobis(succinimidyl proprionate). Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

supplied Na_2ATP, MgATP, ADP (vanadate-free),Ado*PP*[NH]*P*, *p*-nitrophenyl phosphate, Tris, ouabain, quercerin and oligomycin. Brij-58 (Sigma) was twice precipitated from ether before use. 2-Deoxy-ATP, inosine triphosphate, cytidine triphosphate, guanosine triphosphate and uridine triphosphate were obtained from Terochem Laboratories. Calbiochem supplied formycin-A triphosphate and sodium deoxycholate. Nu-Chek-Prep supplied fatty acids. [^{14}C]Palmitic acid was obtained from New England Nuclear; DCCD was obtained from Eastman Kodak; fatty acid-poor bovine serum albumin was supplied by Miles Research Laboratories; Sepharose 6B, DEAE-Sephacel and calibration standards for gel filtration chromatography and gel electrophoresis were obtained from Pharmacia Fine Chemicals, and dithiobis(succinimidyl proprionate) was obtained from Pierce Chemical Company. Samples of aurovertin were gifts generously supplied by Gottfried Schatz (University of Basel, Switzerland) and Dr. Michel Satre (Centre for Nuclear Studies, Grenoble, France). Efrapeptin and leucinostratin were gifts generously supplied by Dr. R. Hamil (Eli Lily Co.).

Methods

The method for culturing the organism *A. laidlawii* B, as well as the procedures for membrane isolation, analysis of membrane lipids, protein determination and assay of ATPase and *p*-nitrophenylphosphatose activities, were essentially the same as those previously used in this laboratory [3]. Brij-58 was determined by an assay of its poly(ethylene glycol) content [9]. The lipid content of the ATPase preparations was determined from the radioactivity contained in a sample purified from organisms cultured on an equimolar mixture of myristic acid and [^{14}C]palmitic acid under conditions of inhibited fatty acid biosynthesis [5]. The purified ATPase was reconstituted into lipid vesicles by extensive dialysis, after relipidation by methods similar to those described by Ottolenghi [10]. Polyacrylamide gel electrophoresis was performed in the gel systems described by Weber and Osborn [11] and Laemmli [12]. Sedimentation equilibrium and sedimentation velocity studies were performed in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. Visible and ultraviolet spectra were recorded with a Beckman DU-8 computing spectrophotometer.

Purification of the ATPase

Membranes prepared from 8 litres of late log culture (approx. 300–350 mg membrane protein) were washed with distilled water and resuspended in 200 ml distilled water. The suspension was warmed to 37°C and mixed with an equal volume of solubilizing buffer (16% (w/v) ethylene glycol, 3 mg/ml Brij 58, 1 mg/ml sodium deoxycholate, 10 mM Tris-HCl (pH 7.6)). The mixture was immediately treated with the protease inhibitor PMSF (100 mM in DMSO) so as to obtain a final PMSF concentration of 100 μM, and incubated at 37°C for 10 min. The non-solubilized material was sedimented by centrifugation at 100 000 × *g* for 1 h at room temperature. The supernatant was then concentrated 8–10-fold on a Diaflo XM300 ultrafiltration membrane, applied to a column of Sepharose 6B (5 cm × 90 cm) and eluted at room temperature with elution buffer A (4% (w/v) ethylene glycol/100 μg/ml Brij 58/33 μg/ml sodium deoxycholate/1 mM sodium azide/10 mM sodium chloride/10 mM Tris-HCl (pH 7.6)) at a flow rate of 30 ml/h. The fractions containing ATPase activity from two such columns were concentrated 25-fold (to approx. 16 ml) on a Diaflo XM300 membrane and incubated with one-fifth of its volume of solubilization buffer for 5 min at 37°C. After cooling to room temperature, the sample was applied to a Sepharose 6B column (1.6 cm × 90 cm) and eluted with elution buffer A at room temperature at a flow rate of 10 ml/h. The fractions containing ATPase activity were pooled and brought up to 60 mM NaCl by the addition of 1 M NaCl and slowly (approx. 10 ml/h) loaded on a DEAE-Sephacel column (1.6 × 10 cm) that was preequilibrated with elution buffer A. The column was then washed with 3 column volumes of elution buffer A containing 110 mM NaCl and the ATPase activity was eluted with elution buffer A containing 150 mM NaCl at a flow rate of 10 ml/h. The ATPase-containing fractions were pooled and concentrated to 3 ml on a Diaflo XM300 membrane, applied to a Sepharose 6B column (1.5 cm × 110 cm), and eluted at room temperature with elution

buffer B (4% (w/v) ethylene glycol/100 μg/ml Brij 58/33 μg/ml sodium deoxycholate, 1 mM sodium azide/170 mM sodium chloride/10 mM Hepes-NaOH (pH 7.6)) at a flow rate of 5 ml/h. The ATPase eluting from the column was stored in elution buffer at room temperature.

Crosslinking of the ATPase subunits with dithiobis(succinimidyl proprionate)

Crosslinking of the ATPase subunits was carried out at room temperature for 20 min, at a protein concentration of 100 μg/ml. The protein was crosslinked with 40 μM DTSP in elution buffer B. The reaction was stopped by the addition of 1 M Tris-HCl (pH 8.0). For two-dimensional gel electrophoresis, the crosslinking reagent was cleaved by subjecting the crosslinked species to electrophoresis in the second dimension through an agarose bridge containing 50 mM β-mercaptoethanol.

Results

Solubilization

Most of the ATPase activity was incorporated into micellar complexes when the crude membranes were incubated with the detergent mixture under the conditions described. When the non-ionic detergent Brij-58 was used alone, some 30–50% of the ATPase activity was sedimentable by a 1-h centrifugation at 100000 × *g*. This problem of incomplete solubilization was overcome by the inclusion of small amounts of ionic detergents in the incubation mixture at levels below their critical micellar concentrations. Of the ionic detergents tested (cholate, deoxycholate, SDS, CTAB), deoxycholate was found to be the least deleterious to the ATPase. Under the conditions reported here, 55% of the protein, 80% of the lipid and 86% of the ATPase activity present in the crude membrane remained in the supernatant after centrifugation at 100000 × *g* for 1 h (Table I).

Gel filtration chromatography

The solubilized protein and lipid were complexed into detergent micelles which did not permeate a Diaflo XM300 ultrafilter and we were unable to concentrate the 100000 × *g* supernatant to a high protein concentration without concentrating the detergent to levels at which the enzyme was inactivated. Consequently, it was necessary to concentrate the 100000 × *g* supernatant to a detergent concentration near the limiting tolerance of the enzyme (approx 10–12 mg/ml) and the relatively dilute protein concentrate (approx. 3–4 mg/ml) was then fractionated on a large column of Sepharose 6B. The ATPase activity eluted as a small shoulder of E-280 absorbing material just ahead of the main peak of protein and lipid (Fig. 1A). The ATPase activity which eluted behaved as one peak when directly applied to a Sepharose 6B column, even though SDS-gel electrophoresis revealed some 20 polypeptide bands, which showed some variability as regards

TABLE I

PURIFICATION OF THE $(Na^+ + Mg^{2+})$-ATPase FROM *A. LAIDLAWII* B

	Crude membranes	Membrane/detergent incubation mixture	$10^5 \times g$ supernatant	1st Sepharose 6B gel filtration	2nd Sepharose 6B gel filtration	DEAE-Sephacel ion exchange	3rd Sepharose 6B gel filtration
Specific activity	19	18	34	90	120	160	180
Recovery of ATPase activity (%) [a]	100	95	90	70	70	30	80
Recovery of protein (%) [a]	100	100	55	26	52	22	65
Lipid content [b]	1.06	1.06	1.54	0.54	0.34	0.23	0.09
Recovery of lipid (%)	100	100	80	9	33	15	25

[a] Recoveries refer to ATPase-associated material obtained for each step in the procedure. Overall recovery of ATPase activity is approx. 10%.

[b] Lipid content quoted in units of μmol glycerolipids/mg protein.

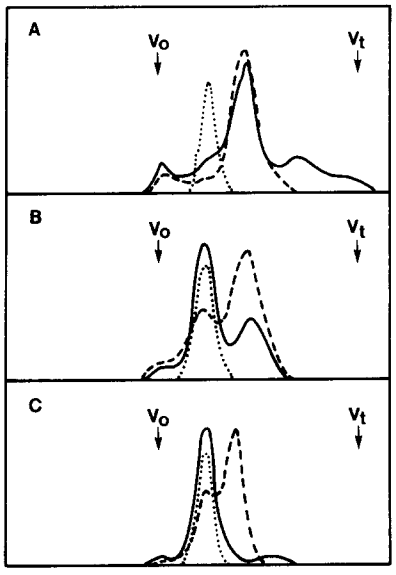

Fig. 1. Sepharose 6B gel filtration chromatography of Brij-58-solubilized *A. laidlawii* ($Na^+ + Mg^{2+}$)-ATPase. (A), first gel filtration; (B) second gel filtration; and (C) third gel filtration. Symbols are ———, A_{280} (protein concentration); ·····, ATPase activity; and (- - - - - -), glycerolipid concentration (cpm).

the number, type and proportions from one preparation to the next. This suggests that the ATPase was intercalated into detergent micelles which contained a heterogeneous distribution of other proteins. Consequently, an attempt was made to redistribute those proteins into other detergent micelles by re-extracting the preparation with more detergent before refractionation on Sepharose 6B. After re-extraction with detergent as described under Materials and Methods, the sample eluted off Sepharose 6B as shown in Fig. 1B. It is clear that a significant fraction of the lipids and non-ATPase protein was redistributed away from the ATPase activity. This was reflected by there being fewer bands on an SDS-gel profile and a 33% increase in the specific activity of the preparation obtained (Table I).

DEAE-Sephacel ion exchange chromatography

Initially, linear salt gradients (50–500 mM NaCl) were employed to elute the ATPase activity bound to DEAE-Sephacel. Such experiments were characterized by a low recovery of ATPase activity (approx. 5%) and the elution of a broad smear of protein and ATPase activity. The first fractions of ATPase activity eluted after a small smear of protein material and yielded five polypeptide bands when analysed by SDS-polyacrylamide gel electrophoresis. These bands were contaminated by other proteins in the later fractions. The use of a shallower salt gradient did not improve the resolution of these five bands and the later contaminants, but it lowered the ratio of ATPase-containing material to the preceding protein material while drastically reducing the recovery of ATPase activity. The protein eluting before the ATPase activity was found to contain two polypeptide bands (M_r 68 000 and 35 000), which co-migrated with two of the bands present in the ATPase-containing fractions. This suggested that the ATPase is a complex of subunits which were being dissociated under the conditions decribed above. The use of step gradients apparently circumvented this problem and increased the recovery of ATPase activity. A step gradient of 110–170 mM NaCl enabled a 60% recovery of ATPase activity; however, the preparation obtained was contaminated with some of the later proteins. The step gradient of 110–150 mM NaCl described under Materials and Methods was the optimal gradient for maximizing ATPase recovery (approx. 30%, see Table I), while minimizing contamination from other proteins and subunit dissociation. Since contamination of the active ATPase by inactive dissociated subunits was still probable after the above procedure, the preparation was further fractionated on a Sepharose 6B gel filtration column. Here it was necessary to elute the ATPase at a salt concentration higher than that used to elute the ATPase from the ion-exchange column, since any dilution of the salt concentration destabilized the micellar complex and resulted in some aggregation of the protein. The elution profile shown in Fig. 1C clearly demonstrates that the ATPase eluted as a single protein peak, ahead of the smaller peak of dissociated subunits and most of the lipid not complexed with the ATPase.

Molecular characterization of the ATPase complex

The purified ATPase complex eluted off Sepharose 6B with the $K_{av}(0.3)$ expected of a glubular protein of molecular weight 900 000–950 000. The observed K_{av} probably reflects primarily the shape

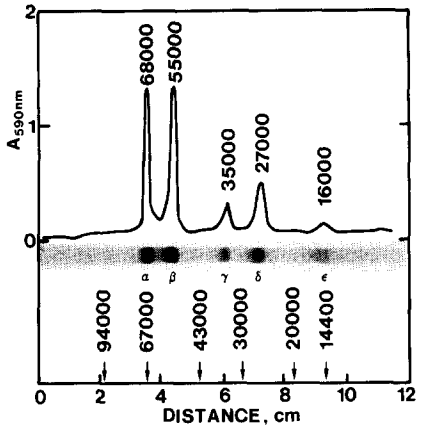

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified *A. laidlawii* ($Na^+ + Mg^{2+}$)-ATPase in the Weber and Osborn system. The band are designated, α, β, γ, δ or ε in order of decreasing molecular weight. The section above the gel is a scan of the Coomassie brilliant blue staining intensities at 590 nm.

properties of the micellar complex, since it did not change during purification even though the protein composition of the micelles was obviously altered. On analysis, 1 mg of protein was found to be associated with 450–650 μg of Brij-58, 88 ± 6 nmol of glycerolipid and trace quantities of deoxycholate and carotenoids. The presence of carotenoids was indicated by the absorption spectrum, which exhibited three small maxima between 420 and 490 nm. Quantification of these carotenoids was not feasible owing to the heterogeneity of such pigments in *A. laidlawii* membranes [13]. Calculations using some reported extinction coefficients [13] indicated that there was less than 1 nmol carotenoids per mg protein. From the ultraviolet absorption spectrum, values of 24.7 and 1.84 were calculated for $A_{280}^{1\%,1cm}$ and A_{280}/A_{260} for the protein.

In analytical ultracentrifugation studies, the ATPase sedimented ideally with a sedimentation coefficient ($s_{20,w}$) of 14.5 ± 0.2 S. Sedimentation equilibrium studies failed to give any estimates of the molecular weight of the complex, since it did not reach equilibrium under the ionic conditions needed to stabilize the ATPase complex. The sam-

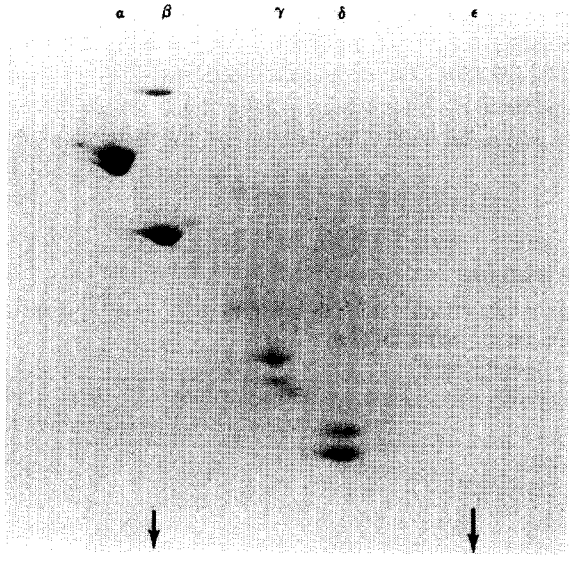

Fig. 3. Two-dimensional SDS polyacrylamide gel electrophoresis of the purified *A. laidlawii* ($Na^+ + Mg^{2+}$)-ATPase, incorporating a Weber and Osborn system for the first dimension and a Laemmli system for the second dimension. The positions of the bands in the first dimension are indicated at the top of the gel. The arrows show the position of the ε subunit and the fragment which ran at the dye front.

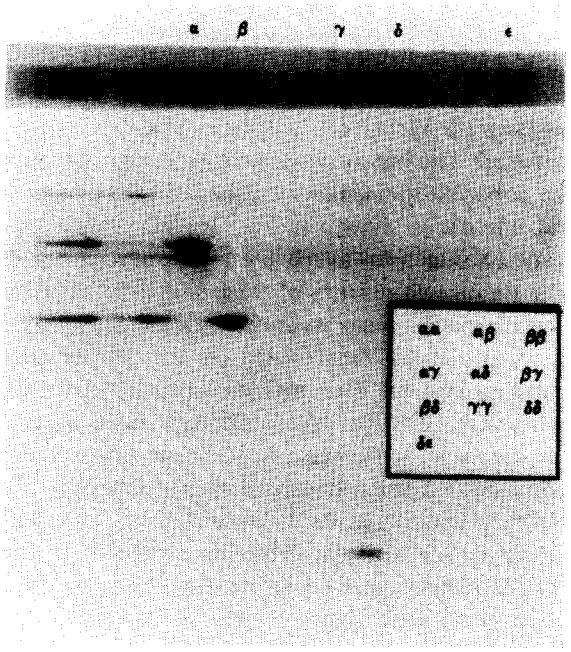

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified *Acholeplasma laidlawii* B ATPase crosslinked with DTSP. The top gel is a Weber and Osborn first-dimensional gel. The bottom gel shows the banding pattern in a second-dimensional Laemmli gel after cleavage of the crosslinker by electrophoresis through an agarose bridge loaded with β-mercaptoethanol. The insert shows the subunit composition of the crosslinked species.

TABLE II

ASSIGNMENTS OF THE SUBUNIT COMPOSITION OF THE CROSSLINKED SPECIES FORMED WITH DITHIOBIS(SUCCINIMIDYL PROPRIONATE)

Apparent M_r	Subunit designation
140000	$\alpha\alpha$
120000	$\alpha\beta$
110000	$\beta\beta$
100000	$\alpha\gamma$
95000	$\alpha\delta$
90000	$\beta\gamma$
82000	$\beta\delta$
70000 [a]	$\gamma\gamma$
55000 [a]	$\delta\delta$
45000	$\delta\varepsilon$

[a] These bands overlapped with the α and β bands, respectively. Molecular weights were assigned from the banding patterns in the second dimension

ple did reach equilibrium under low-salt conditions (approx. 10 mM NaCl), whereupon it behaved as a mixture containing mainly species of M_r approx. 600000 and a smaller population of species of M_r approx. 100000.

The purified ATPase was resolved into five bands by a Weber and Osborne SDS polyacrylamide gel. The bands designated α–ε migrated with the apparent molecular weights and relative staining intensities shown in Fig. 2. The sample was resolved into nine bands by a Laemmli SDS gel. The staining intensities of these suggested that the observed differences were the product of multiple banding of the bands seen in the Weber and Osborne system. This was confirmed by two-dimensional SDS gel electrophoresis in a system incorporating a Weber and Osborne first dimension and a Laemmli second dimension (Fig. 3). Multiple banding in the Laemmli gel was apparent for all bands with the probable exception of the ε-band, which migrated at the dye front. Furthermore, there were signs of anamolous behavior of the β-band, since it migrated as three bands in the Laemmli system. Apart from the main band on the diagonal, there was a band which migrated more slowly than the α-band and a 'fragment' migrating at the dye front. These observations raised questions about the probability of the copurification of the ATPase with other proteins. This aspect was investigated by crosslinking studies using the cleavable reagent dithiobis(succinimidylproprionate). The results shown in Fig. 4 were obtained by an analysis of the crosslinked sample in a Weber and Osborn first-dimensional gel followed by a Laemmli second-dimensional gel after cleavage of the crosslinker. The first-dimensional gel shows a variety of crosslinked species migrating with apparent molecular weights ranging from 46000 to 140000. A combination of the apparent molecular weights and banding patterns observed in the second-dimensional gel enabled the assignment of the crosslinked species shown in Table II. It is clear that all the bands resolved by the gel electrophoresis systems used were involved in crosslinked species with at least one other subunit. Since the experimental conditions of low concentrations of protein and crosslinker minimize the probability of intermolecular crosslinking, these observations support the view that the ATPase is an assembly of all the bands resolved by gel electrophoresis. A close inspection of the gel revealed that the α and β subunits derived from the crosslinked species were resolved as multiple bands by the Laemmli gels and that the components of these subunits did not crosslink to other subunits independently. In contrast, the γ and δ subunits derived from the crosslinked species showed some tendency to migrate as single bands which corresponded to the components of the doublets de-

TABLE III

SUBSTRATE SPECIFICITY OF THE $(Na^+ + Mg^{2+})$-ATPase FROM *A. LAIDLAWII* B

Substrate	Specific activity (μmol P_i/mg protein per h)
ATP	210
2-Deoxy-ATP	170
ITP	125
GTP	99
UTP	55
CTP	0
Formycin-A triphosphate	91
p-Nitrophenyl phosphate [a]	1.9

[a] Specific activity quoted in units of μmol *p*-nitrophenol/mg protein per h. The *p*-nitrophenol phosphatase in the crude membranes was 4.5 units.

TABLE IV

EFFECT OF ATPase INHIBITORS ON THE ACTIVITY OF *A. LAIDLAWII* B ATPase

Inhibitor	Concentration	Percentage inhibition
Sodium azide	$1 \cdot 10^{-3}$ M	0
Aurovertin	$2 \cdot 10^{-5}$ M	0
Quercetin	$1 \cdot 10^{-4}$ M	0
Oligomycin [a]	100 μg/ml	15
DCCD	$5 \cdot 10^{-4}$ M	22
Efrapeptin	1 mg/ml	15
Leucinostatin [b]	$5 \cdot 10^{-4}$ M	50
Ouabain	$1 \cdot 10^{-3}$ M	0
Sodium orthovanadate [c]	$5 \cdot 10^{-4}$ M	50

[a] ATPase activity in native membranes was slightly more oligomycin-sensitive. 25% inhibition was detected at concentrations near the limiting aqueous solubility of oligomycin.

[b] 90% inhibition was observed at $1.4 \cdot 10^{-3}$ M leucinostatin. The concentration dependence suggests that simple reversible inhibition does not take place and that the inhibition may be better described by cooperative reversible binding.

[c] 75% of the ATPase was sensitive to vanadate. The residual ATPase activity was insensitive to vanadate concentrations in excess of 10^{-2} M. The concentration-dependence of the vanadate-sensitive fraction was describable by reversible non-competitive inhibition with a K_i of $3.5 \cdot 10^{-4}$ M.

scribed earlier, and these appeared to crosslink to other subunits independently. If the multiple bands resolved by the Laemmli gel were the result of the comigration of structurally different polypeptides, the formation of independent crosslinking patterns with other subunits would be expected. Given this, our data would suggest that the multiple bands described by the α and β subunits are not the result of structurally different polypeptides, while such could be the reason for the splitting of the γ and δ bands in the Laemmli system. This, in turn, suggests that the ATPase may be an assembly of seven polypeptides and not five.

Kinetic characterization of the purified ATPase

The purified ATPase has a specific activity of 195–200 units (μmol P_i/mg protein per h) under the standard assay conditions at 37°C (1 mM ATP/15 mM Mg^{2+}/50 mM Na^+ (pH 7.5)). The activity could be enhanced by 25% upon treatment with a sonicated dispersion of phosphatidylcholine and a negatively charged phospholipid. The specific activity of the relipidated preparation represents a 14-fold purification over that of the crude membranes and suggests that the ATPase proteins account for no more than 7% of the protein present in these membranes.

At low ATP concentrations (approx. 2 mM), the hydrolysis of ATP was describable by Michaelis-Menten kinetics with a K_m of $1.65 \cdot 10^{-4}$ M. Higher concentrations of ATP progressively inhibited the enzyme, which was completely inhibited by 10 mM ATP. In comparison with other nucleoside triphosphates, the hydrolysis of ATP proceeded at a faster rate at similar substrate concentrations (Table III) and considerable sensitivity to substrate modification was indicated. The specific activity of the *p*-nitrophenolphosphatase activity was one-half that of the crude membranes (Table III) and this activity did not co-purify with the ATPase activity. This observation obviously does not rule out the probability of the ATPase having some *p*-nitrophenolphosphatase activity, but it clearly indicates that the majority of the *p*-nitrophenolphosphatase activity of the native membranes is not associated with the ATPase. Adenosine diphosphate and the nonhydrolysable ATP analog, Ado*PP*[NH]*P*, were both competitive inhibitors, with K_i values of $2 \cdot 10^{-3}$ M and $6.8 \cdot 10^{-5}$ M, respectively.

In inhibition studies, both the native membrane and the purified ATPase were found to be insensitive to a variety of inhibitors known to have some specificity of F_1-F_0-type ATPases (Table IV). Such inhibitors either had no effect on ATP hydrolysis (azide, quercetin, aurovertin) or were poor inhibitors even at concentrations considerably higher than that generally used for F_1-F_0 ATPases. Inhibition by DCCD and oligomycin was observed only at concentrations near the limiting aqueous solubilities of the reagents. Both the membrane-bound and purified ATPase were relatively insensitive to leucinostatin and efrapeptin, which are potent inhibitors of phosphoryl transfer in mitochondria [14]. ATPase activity was also extremely insensitive to inhibitors of eukaryotic plasma membrane-bound ATPases. Thus ATP hydrolysis was unaffected by ouabain, which specifically inhibits the ($Na^+ + K^+$)-ATPase of animal plasma membranes. Inhibition by orthovanadate was not complete (Table IV) and occurred at near millimolar

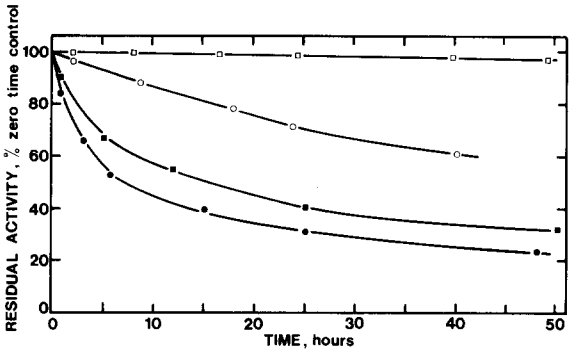

Fig. 5. Time-course of inactivation of the *A. laidlawii* B ATPase at 0°C. Symbols are □———□, purified relipidated ATPase; ○———○, Brij-58 solubilized membrane protein; ■———■, ATPase from first Sepharose 6B gel filtration; ●———●, ATPase from second Sepharose 6B gel filtration.

concentrations, whereas most plasma membrane ATPases are completely inhibited at micromolar concentrations of this reagent [15–17]. The inhibition of the purified ATPase by some irreversible inhibitors was similar in its sensitivity characteristics to that reported for the ATPase in the crude membranes [3].

Unlike the crude membranes, the ATPase activity of the solubilised preparations is cold-labile (see Fig. 5). The cold stability of such preparations varied during purification and was apparently related to the stability of the detergent micelles into which the enzyme was intercalated. The cold-inactivation process probably increased the probability of subunit dissociation, since the formation of the dissociation products was more apparent when ion-exchange chromatography was carried out in the cold. The stability of the ATPase preparations could be enhanced by increasing the polarity of the media or by treatment with phospholipids. The latter usually resulted in the restoration of most of the lost activity provided that no more than 60% of the initial activity was lost. Once relipidated, the purified ATPase was kinetically indistinguishable from the native membrane-bound form.

Discussion

The solubilization procedure described here employed low protein and detergent concentrations which apparently enable some selectivity with respect to the ATPase. The behavior of the solubilized material on the Diaflo XM300 ultrafilter and the Sepharose 6B column clearly showed that the solubilized protein was incorporated into large micellar complexes of detergent, protein and lipid. Here, the inclusion of small amounts of an ionic detergent was used to form charged, mixed-detergent micelles such that the charge repulsion would reduce the probability of aggregation. Gel filtration chromatography showed that the solubilized membrane protein was mainly distributed between two populations of micelles of which the ATPase-containing peptides eluted with a larger Stokes' radius. Our observations suggest that those micelles also contained a heterogeneous distribution of other proteins. The formation of such particles was suggested by a previous attempt to fractionate solubilized membrane proteins from this organism [18]. The purification of the ATPase from micellar particles containing such a heterogeneous population of other proteins required a redistribution of the latter away from the ATPase, which could then be separated by gel-filtration chromatography owing to the larger Stokes' radius of the ATPase-containing particles. This redistribution was achieved by a combination of re-extraction with detergent and ion-exchange chromatography. The successful elution of the ATPase from a DEAE ion-exchange column described here is in marked contrast to previous attempts in which the applied protein appeared to be irreversibly bound [2,7]. In one instance this could be ascribed to the use of a deoxycholate-solubilized preparation in which the membrane protein was incorporated into negatively charged detergent micelles, with the result that the high negative charge density made their desorption from the column very difficult. The problems associated with the use of sonicated dispersions of *A. laidlawii* membrane protein for DEAE anion-exchange chromatography [2] may have been the result of aggregation or, as suggested later [8], inadequate elution conditions.

The different banding patterns of the ATPase on the two gel systems used raised questions about the exact subunit composition of the ATPase and the purity of the preparation. However, the cross-linking experiments, along with our inability to obtained enzymatically active preparations containing fewer polypeptide bands, provided strong evidence that all the bands resolved by gel electro-

phoresis are associated with the ATPase (i.e., the ATPase preparation is protein pure). The crosslinking experiments also suggested that the observed multiple banding of the α- and β-subunits in the Laemmli gel system may be artifactual, given the persistence of such multiple banding amongst the crosslinked species involving these subunits. Given the time required to purify the ATPase (approx. 10 days), the probability of degradation artifacts cannot be excluded. The crosslinking experiments also suggested that the γ- and δ-subunits resolved on the Weber and Osborn gels may each be two polypeptides (i.e., the ATPase may be an assembly of seven subunits and not five). These unresolved issues preclude any meaningful estimates of the protein molecular weight, the exact subunit composition and stoichiometry as well as the probable arrangement of the subunits. While it is clear from the crosslinking experiments that the ATPase must be assembled such that the subunit pairs shown in Table II lie within the crosslinking distance of DTSP (11 Å), it is not feasible to obtain more structural information from such experiments until the above issues are settled. Indeed, the unambiguous resolution of these issues may only be possible when the ATPase is available in quantities which make the purification of the individual subunits for amino acid analyses and sequencing feasible.

The similarity of the kinetic properties of the purified preparation and the membrane-bound form argues against any substantial alteration of the enzyme during purification. The kinetic characterization described here supports earlier studies [2,3] in suggesting that this ATPase is unlike any of the other ATPases that have been described in the literature. This study shows clearly that this ATPase is structurally dissimilar to the $(Na^+ + K^+)$-ATPase of animal cells, the Ca^{2+}-ATPase of sarcoplasmic reticulum and the plasma membrane ATPases of the type found in most prokaryotic microorganisms. The enzyme does show some similarity to the F_1-F_0-type ATPases with respect to its polypeptide composition and instability when stored in the cold at low ionic strength. However, the insensitivity of both the purified and native membrane-bound ATPase to azide ion and the antibiotics tested (especially aurovertin), as well as their insensitivity to DCCD, clearly show that their kinetic behavior is unlike the F_1-F_0-type ATPases. Furthermore, the A_{280}/A_{260} ratio reported here (1.84) is considerably higher than that expected of F_1 particles, for which, on account of tightly bound nucleotides, values of 1.34 to 1.5 are typical [19,20]. Given this, it is clear that the *A. laidlawii* B ATPase is not a typical bacterial F_1-F_0 ATPase from which the F_1 portion has been solubilized and purified, and the low sensitivity of the purified ATPase to inhibitors such as oligomycin and DCCD cannot be ascribed to the loss of an F_1 particle during purification, since such inhibitors also have little effect on the native enzyme in its unperturbed membrane environment.

The decrease in the lipid : protein ratios during purification (Table I) suggests that some delipidation of the ATPase may have occurred. The enhancement of the catalytic activity of the purified preparation by phospholipid is consistent with this hypothesis and suggests that the lipid associated with the purified ATPase may be near the minimum required by this enzyme for full activity. In addition, the sensitivity of the preparation to phospholipid as evidenced by the enhancement of its catalytic activity, the stabilization against cold-inactivation and the reactivation of the cold-inactivated enzyme suggest that the enzyme was purified with its intramembrane domain intact. This was supported by some preliminary crosslinking experiments using photosensitive phospholipids (data not presented) which showed that part of the α subunit of the purified ATPase is buried in the hydrophobic core of the lipid bilayer. This is in marked contrast to the F_1 particle, which does not penetrate the hydrophobic core of the lipid bilayer.

With the purification of this ATPase, it is now possible to reconstitute the enzyme into defined lipid systems amenable to some of the physical techniques available for the study of lipid-protein interactions. But, given the unique properties of this ATPase, it is apparent that such studies should be preceded by further characterization aimed at clearly defining its molecular weight, subunit composition, stoichiometry and geometric arrangement, as well as the subunit(s) which define its active site and intramembrane domain. These characterization studies are currently in progress.

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