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Characterization and Solubilization of the Membrane-Bound ATPase of *Mycoplasma gallisepticum*

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The membrane-bound ATPase of *Mycoplasma gallisepticum* selectively hydrolyzed purine nucleoside triphosphates and dATP. ADP, although not a substrate, inhibited ATP hydrolysis. The enzyme exhibited a pH optimum of 7.0 to 7.5 and an obligatory requirement for divalent cations. Dicyclohexylcarbodiimide at a concentration of 1 mM inhibited 95% of the ATPase activity at 37°C, with 50% inhibition occurring at 22 μM dicyclohexylcarbodiimide. Sodium or potassium (or both) failed to stimulate activity by greater than 37%. Azide (2.6 mM), diethylstilbestrol (100 μg/ml), *p*-chloromercuribenzoate (1 mM), and vanadate (50 μM) inhibited 50, 91, 89, and 60%, respectively. The ATPase activity could not be removed from the membrane without detergent solubilization. Although most detergents inactivated the enzyme, the dipolar ionic detergent *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (0.1%) solubilized approximately 70% of the enzyme with only a minor loss in activity. The extraction led to a twofold increase in specific activity and retention of inhibition by dicyclohexylcarbodiimide and ADP. Glycerol greatly increased the stability of the solubilized enzyme. The properties of the membrane-bound ATPase are not consistent with any known ATPase. We postulate that the ATPase functions as an electrogenic proton pump.

The membrane-bound ATPase of *Mycoplasma gallisepticum* plays a central role in cell volume regulation (25, 34). To understand the mechanism by which the ATPase functions in this manner, the properties of the membrane-bound enzyme have been studied. Of particular relevance to studies of volume regulation is the identification of the ion pumped by the enzyme.

Studies of the ATPase in two related organisms have been reported. Jinks et al. (21) reported a fourfold stimulation of ATPase activity by Na^+ in *Acholeplasma laidlawii* and concluded that the enzyme translocated Na^+ across the membrane. Benyoucef et al. (6) postulated the existence of ATP-dependent Na^+-K^+ exchange in *Mycoplasma mycoides* var. Capri.

Studies reported in this communication demonstrate that the ATPase of *M. gallisepticum* has an obligatory requirement for divalent cations and hydrolyzes purine nucleoside triphosphates and dATP with equal efficiency. The enzyme is quite sensitive to dicyclohexylcarbodiimide (DCCD), azide, diethylstilbestrol, mercurials, and ADP, but is relatively less sensitive to vanadate. The enzyme is only slightly stimulated by monovalent cations. These findings plus other properties of this system are consistent with the hypothesis that this ATPase is a proton pump.

MATERIALS AND METHODS

Preparation of membranes. Membranes were prepared by osmotic lysis after loading with glycerol by the method of Razin and Rottem (31).

Preparation of *M. gallisepticum* and *Escherichia coli* lipids was by the method of Ames (3).

ATPase assay. In a typical assay the reaction mixture (0.5 ml) contained 5 mM NaCl, 5 mM $MgSO_4$ or $MgCl_2$, 0.5 mM dithiothreitol or 2-mercaptoethanol, 50 mM buffer (pH 7.0 to 7.5), and 5 to 100 μg of freshly thawed membrane protein. To start the reaction, 5 mM Na_2-ATP (pH 7.0) was added, and the mixture was incubated for 10 or 15 min. The reaction was stopped by the addition of 0.1 ml of ice-cold 30% (wt/vol) trichloroacetic acid and centrifuged at 12,000 × *g* for 1 min in a Beckman Microfuge. The supernatants were used for the determination of inorganic phosphate by the method of Ames and Dubin (4).

Protein determination. Protein was determined by the method of Peterson (29). In this procedure, proteins were first solubilized with deoxycholate and then precipitated with trichloroacetic acid. Sodium dodecyl sulfate was included during the incubation with Folin-Ciocalteu phenol reagent.

Solubilization of the ATPase activity with dipolar ionic sulfobetaines. Freshly thawed membranes at a final concentration of 1 mg of protein per ml were solubilized with detergent for 30 min at 4°C in the presence of 10 mM Tris (pH 7.4) and 1 mM dithiothreitol. After a sample of the incubation mixture (unfractionated) was removed, the remainder was centrifuged for 60 min at 4°C in a Beckman 50 Ti rotor at 45,000 rpm. The supernatant was removed, and the pellet was suspended in 10 mM Tris (pH 7.4). ATPase activities and protein determinations on the unfractionated, supernatant, and suspended pellet samples were performed as described above.

Materials. Dipolar ionic sulfobetaines (Zwittergents) were obtained from Calbiochem. They are designated by the names "3-*n*," where *n* designates the number of carbon atoms of one substituent group on the nitrogen atom.

Octylglucoside, ouabain, carbonyl cyanide-*m*-chlorophenyl hydrazone, were from Calbiochem. Nucleoside triphosphates, AMP, ADP, dATP, *p*-nitrophenylphosphate, pyruvate kinase, oligomycin (mixture of oligomycin A, B, and C), *p*-chloromercuribenzoate, diethylstilbestrol, and DCCD were from Sigma Chemical Co. Sodium orthovanadate was from Fisher Scientific Co. Efrapeptin was a gift of Lilly Research Laboratories. *Neurospora crassa* plasma membranes were a generous gift of Carolyn W. Slayman.

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RESULTS

General properties of the membrane-bound ATPase. The ATPase activity of washed membranes of *M. gallisepticum* was measured by determining the rate of release of P_i from ATP. The pH optimum for ATP hydrolysis was 7.0 to 7.5 (data not shown). The enzyme was able to hydrolyze the purine nucleoside triphosphates (UTP, ITP, GTP, and ATP) and 2'-dATP with similar efficiency. The enzyme was unable to hydrolyze ADP or *p*-nitrophenylphosphate and hydrolyzed AMP, CTP, and TTP at only 26 to 28% of the rate of ATP hydrolysis. ADP, although itself not a substrate, inhibited ATP hydrolysis about 50% when both were present at a concentration of 5 mM. The rate of ATP hydrolysis could be doubled by removing the inhibiting ADP with phosphoenolpyruvate and pyruvate kinase.

The enzyme has an obligatory requirement for divalent cations in the following order: Mg^{2+} (100%), Co^{2+} (66%), Mn^{2+} (37%), Ca^{2+} (27%), and Ba^{2+} (0%).

Monovalent cation stimulation. Since Na^+-translocating ATPases generally exhibit a marked Na^+ stimulation of ATP hydrolysis in vitro (19, 36), it was important to study the effects of cations on the enzyme of *M. gallisepticum*. Each of the monovalent cations at a concentration of 10 mM showed a very slight stimulation of ATPase activity (Table 1). The presence of both Na and K at concentrations of either 10 or 50 mM resulted in no more than 25% stimulation. To examine the effects of Na^+ and K^+ in greater detail, the ion concentration was varied over a range of several orders of magnitude. Atomic absorption was employed when Na^+ or K^+ was present in the micromolar concentration range. No stimulation of the ATPase activity greater than 37% was observed (Tables 2 and 3). The relatively weak stimulation of enzyme activity by monovalent cations is consistent with nonspecific salt effects.

Inhibitors. A variety of potential inhibitors was examined. Access to both faces of the membrane by the inhibitors was assured by the known leakiness of *M. gallisepticum* membranes prepared in the manner described previously (30, 39). The first inhibitor tested was DCCD, which is known to inhibit the H^+-ATPases of bacteria (16, 32), mitochondria (37), plant roots (23), and fungi (13). DCCD inhibited the ATPase of *M. gallisepticum* in a monotonic manner (Fig. 1), which is consistent with only a single species of inhibitable enzyme. At a concentration of approximately 22 μM, 50% inhibition of the enzyme was observed. At a concentration of DCCD of 1 mM or greater the ATPase activity was inhibited 95% at 37°C (and 86% at 25°C). The ATPase of *A. laidlawii* (21) exhibits a lower affinity for inhibition by DCCD (K_i of 100 μM), whereas that of *M. mycoides* var. Capri shows a similar affinity to that of *M. gallisepticum* (6).

Having demonstrated that the ATPase activity was inhibited by DCCD and that Na^+ and K^+ failed to significantly stimulate the activity, there remained the possibility that a second ATPase, which was DCCD insensitive, was strongly stimulated by Na^+ and thus functioned as a Na^+ pump. Such a cation pump was demonstrated in *Streptococcus faecalis* by Heefner and Harold (17). It was found that the DCCD-insensitive ATPase activity of *M. gallisepticum* was not stimulated by Na^+ or K^+.

Azide is a potent inhibitor for the F_0F_1 ATPase of mitochondria (13) and of several bacteria (11, 32), but has little effect on the membrane-bound ATPase of *A. laidlawii* (21) or the purified enzyme from this species (9, 24). Potassium azide inhibited the ATPase of *M. gallisepticum* by 50% at a concentration of approximately 2.6 mM and 69% at 10 mM.

Orthovanadate in the micromolar concentration range

TABLE 1. Effect of salts of monovalent cations on ATPase activity[a]

Salt (10 mM)	ATPase activity[b]	
	nmol of P_i/min per mg	% Increase
None	172	0
NaCl	235	37
KCl	202	17
LiCl	182	6
RbCl	192	12
Choline-chloride	214	24
NH_4Cl	208	21
CsCl	193	12
NaCl, KCl	215	25
NaCl (49 mM), KCl (49 mM)	208	21

[a] Membranes (50 μg protein) were incubated in triplicate (0.5 ml) at 37°C with 5 mM $MgCl_2$, 50 mM Tris (pH 7.4), 0.5 mM 2-mercaptoethanol, 5 mM MgATP, and the salts indicated above. NH_4Cl stock was adjusted to pH 7.4 using Tris base.

[b] Mean values of two separate experiments.

TABLE 2. Effect of Na^+ on ATPase activity[a]

NaCl	ATPase activity[b]	
	nmol of P_i/min per mg	% Increase
8.7 μM	176	0
49 μM	201	14
100 μM	204	16
5 mM	215	22
10 mM	228	30
50 mM	241	37
100 mM	186	6
150 mM	176	0

[a] The membrane preparation was dialyzed against 3.5 liters of 2.5 mM Tris (pH 7.4) plus 1 mM 2-mercaptoethanol for 19 h at 4°C with three changes of medium to remove traces of Na^+. The enzyme (50 μg of membrane protein) was incubated in triplicate (final volume, 0.5 ml) with 5 mM $MgCl_2$, 50 mM Tris (pH 7.4), 0.5 mM 2-mercaptoethanol, 5 mM MgATP, and various concentrations of NaCl at 37°C. The Na^+ concentration was determined by atomic absorption spectroscopy (Perkin-Elmer 5000). K^+ contamination was 2 μM or less.

[b] Mean values of triplicate incubations.

TABLE 3. Effect of K^+ on ATPase activity[a]

KCl[b]	Na^+ Contamination[b] (μM)	ATPase activity[c]	
		nmol of P_i/min per mg	% Increase
1.3 μM	8.7	174	0
10 μM	8.7	216	24
100 μM	8.8	227	31
5 mM	9.8	185	24
10 mM	10.8	212	22
49 mM	19.3	181	5
100 mM	29.8	169	−3

[a] Incubation conditions are identical to that of Table 2.

[b] K^+ and Na^+ concentrations were determined by atomic absorption spectroscopy.

[c] Mean values of triplicate incubations.

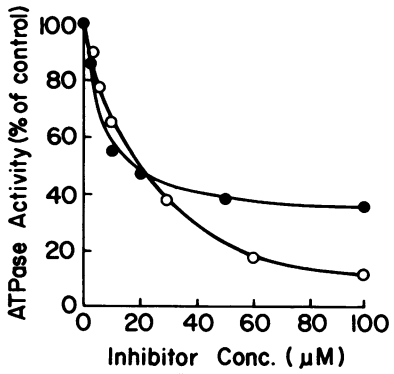

FIG. 1. Inhibition of ATPase by DCCD and vanadate. In the DCCD experiment (○), enzyme (650 μg of membrane protein) was added to a series of tubes containing different concentrations of DCCD (final volume, 30 μl). After incubation of these tubes (in duplicate) for 15 min, 5 μl (in duplicate) was diluted into a total volume of 500 μl of ATPase assay mixture. Data points are the mean values of two experiments. In the vanadate experiment (●), enzyme (200 μg of membrane protein) prepared without reducing agents was incubated (in duplicate) for 15 min at 37°C in 50 mM Tris (pH 7.4)–5 mM $MgCl_2$–5 mM KCl plus orthovanadate. The enzyme assay was initiated by the addition of Na_2-ATP (5 mM). Each day a new 0.2 M stock of sodium orthovanadate was prepared (pH 12.5). Immediately before the experiment, the stock vanadate was diluted 10-fold or 100-fold and boiled for 2 min before addition to membranes. Data points are mean values of two experiments.

inhibits several of the membrane-bound ATPases that involve a phosphorylated intermediate such as Na^+,K^+-ATPase (8), the K^+-ATPase of bacteria (20, 27), and the H^+-ATPase of fungi (13). On the other hand it has little or no effect on the F_0F_1 ATPases (27). Benyoucef et al. (6) found that 500 μM vanadate failed to inhibit the ATPase of *Mycoplasma mycoides* var. Capri, whereas Lewis and McElhaney (24) reported that 500 μM vanadate inhibited the purified ATPase of *A. laidlawii* by 50%. In the experiments on *M. gallisepticum* reported here with vanadate, reducing agents were omitted from the membrane preparation and from the ATPase assay because of the need to maintain vanadate in the necessary redox state. In addition, K^+ was included because of the known ability of K^+ to stimulate vanadate inhibition of the Na^+,K^+-ATPase (27). In each experiment plasma membranes of *N. crassa*, known to possess a vanadate-sensitive ATPase (13), were used as a control to assure that the inhibitor was active. As demonstrated in Fig. 1, vanadate inhibited the *M. gallisepticum* ATPase. At inhibitor concentrations of 100 μM or more approximately 40% of the activity remained vanadate insensitive.

Ouabain, the classical inhibitor of the animal cell Na^+,K^+-ATPase (12), does not inhibit the membrane-bound ATPases of *M. mycoides* var. Capri (6), *A. laidlawii* (35), or the purified enzyme of *A. laidlawii* (9, 24). The ATPase of *M. gallisepticum* is also ouabain insensitive (Table 4).

Oligomycin inhibits the mitochondrial F_1F_0 ATPase and, at high concentrations, the Na^+,K^+-ATPase (38), but not the bacterial F_0F_1 ATPase (22). Oligomycin (100 μg/ml) inhibited the *A. laidlawii* purified ATPase by only 15% (24) and also failed to inhibit the *M. gallisepticum* activity (Table 4). Efrapeptin inhibits F_0F_1 ATPases (10, 17, 22), but failed to inhibit the ATPase in *M. gallisepticum* (Table 4).

Diethylstilbestrol is known to inhibit the fungal plasma membrane H^+-ATPase (13), the F_0F_1 ATPase of *S. faecalis* (17), the plasma membrane ATPase of higher plants (5), and various transport ATPases of animal cells (18). Diethylstilbestrol (100 μg/ml) inhibited the *M. gallisepticum* ATPase by 91% (Table 4).

In agreement with Rottem and Razin (35), the ATPase of *M. gallisepticum* was inhibitable by mercurials such as p-chloromercuribenzoate. The ATPase of *A. laidlawii* is also sensitive to mercurials (21, 35).

Solubilization of ATPase activity. Since the characterization of the membrane-associated ATPase revealed some unique properties, it was of interest to solubilize the enzyme for future purification and reconstitution.

It has long been known that the ATPase activity of *A. laidlawii* and various *Mycoplasma* spp. could not be removed from the membrane by simple washing in low-ionic-strength media with EDTA (28, 35), in contrast to the F_1F_0 ATPases of most bacteria (11). Variation of ionic strength or pH or the addition of EDTA failed to extract the activity

TABLE 4. Effect of potential inhibitors on ATPase activity[a]

Substance	Concn	% Inhibition of ATPase
Ouabain	150 μM	2
	1 mM	6
Oligomycin	100 μg/mg of protein	0
Efrapeptin	25 μg/ml	4
CCCP[b]	10 μM	3
Diethylstilbestrol	100 μg/ml	91
pCMB[c]	1 mM	89

[a] For each inhibitor (except *p*-chloromercuribenzoate), 50 μg of membrane protein was incubated at 37°C in a final volume of 0.5 ml containing 5 mM $MgCl_2$, 5 mM NaCl, 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0), 5 mM ATP (pH 7.0), 1 mM 2-mercaptoethanol, and the inhibitor. For all compounds, duplicate determinations were made.

[b] CCCP, Carbonyl cyanide-*m*-chlorophenyl hydrazone.

[c] For *p*-chloromercuribenzoate (pCMB), 250 μg of membrane protein prepared without reducing agents was incubated for 15 min at 37°C with 1 mM *p*-chloromercuribenzoate. A sample was then diluted 100-fold into an ATPase assay mixture identical to the above.

TABLE 5. Solubilization of ATPase activity with dipolar ionic sulfobetaines[a]

Detergent			% of protein solubilized	% of initial enzyme[b]			Supernatant sp act (nmol/min per mg)[c]
Name	CMC[d]	Conc (%, wt/vol)		Unfract	Sup	Pellet	
3-14	0.39	0.05	41	59	16	15	137
		0.10	38	49	5	14	51
		0.19	27	9	0	16	0
3-12	3.09	0.05	10	85	12	86	415
		0.10	21	66	45	19	744
		0.20	33	40	17	7	173
3-10	39	0.10	10	89	4	68	158
		0.50	12	67	8	71	267
		1.00	27	39	25	13	360
3-08	Large	0.10	5	82	5	74	373
		0.49	6	92	4	91	240
		0.98	5	86	4	78	351

[a] See the text for the detailed procedure.

[b] The ATPase activity of the membranes before treatment with detergent was taken as 100%. Abbreviations: unfract, detergent-treated membranes, but unfractionated; Sup, supernatant after centrifugation.

[c] Specific activity of detergent-free membranes was 345 nmol/min per mg.

[d] CMC, Critical micellar concentration.

(data not shown), suggesting that the enzyme is an integral membrane protein.

Attempts were made to solubilize the enzyme in active form by using detergents such as cholate, Triton X-100, and Nonidet P-40, but the enzyme was rapidly inactivated. When octylglucoside (0.88%, wt/vol) was used to extract membranes (1 mg of protein per ml) for 30 min at 4°C, it inactivated 45% of the initial activity and solubilized 80% of the remaining activity with a 1.4-fold increase in the specific activity. The addition of 1 mM EDTA, 2.5 mM ATP, 30% (wt/wt) glycerol, or *E. coli* phospholipid during the solubilization failed to prevent inactivation or increase the specific activity of the octylglucoside-solubilized enzyme.

A series of dipolar ionic sulfobetaines of various alkyl chain lengths (14) was then tested. Zwittergent 3-12 solubilized the ATPase activity, with at least a twofold increase in specific activity and only 20 to 40% inactivation (Table 5). The addition of 24% (wt/wt) glycerol to the dipolar ionic sulfobetaine-solubilized supernatant resulted in preservation of solubilized activity for at least two weeks (Table 6) with retention of DCCD sensitivity (data not shown). The optimal protein concentration for solubilization (with 0.1% [wt/vol] Zwittergent 3-12) was found to be 1 to 2 mg/ml. The membrane-bound and solubilized ATPase activities exhibited nearly identical inhibition by DCCD and ADP (Table 7), indicating that the solubilized enzyme retained the properties of the membrane-associated form. The solubilized enzyme could be concentrated up to fivefold by ultrafiltration without loss of activity. The properties of the dipolar ionic detergent-solubilized ATPase are ideal for purification attempts.

DISCUSSION

The evidence presented here and in other investigations from this laboratory (25, 26, 34) is consistent with the view that the *M. gallisepticum* ATPase functions as an electrogenic H^+ pump, a physiological process found in virtually all microorganisms (11). The evidence for this conclusion can be summarized as follows. (i) The energized membrane of *M. gallisepticum* generates a proton motive force consisting of a membrane potential (inside negative) and a pH gradient (inside alkaline) (34). (ii) DCCD inhibits the ATPase 95% (Fig. 1) and collapses both the ΔpH and the $\Delta\psi$ (25). (iii) Monovalent cations have very little effect on the ATPase activity (Tables 1 through 3).

Of special interest is the question of monovalent cation effects on the ATPase, since Na^+-translocating ATPases always show a marked Na^+ stimulation of ATP hydrolysis in vitro. A 5- to 10-fold stimulation of ATPase activity has been observed for the Na^+,K^+-ATPase of animal cells (36) and an Na^+-ATPase in bacteria (19). In H^+-translocating ATPases, on the other hand, nonspecific salt effects of several cations are frequently observed (1, 2, 7, 33). For example, monovalent cations stimulate (20 to 100%) ATPases known to translocate H^+ such as those found in *N. crassa* (7) and bacteria (1, 15, 33).

The effect of inhibitors on the ATPase is not the same as that found with such enzymes in other organisms. The *M. gallisepticum* enzyme is inhibited by DCCD and azide, two classical inhibitors of the F_0F_1 H^+-translocating ATPases (16, 32). However, DCCD blocks the *M. gallisepticum* at a concentration (K_i, 22 μM) somewhat greater than that required for the F_0F_1 ATPase, although at a similar concentration required for the ATPase of yeast and fungi (13). Although vanadate inhibits the *M. gallisepticum* enzyme, the concentration required for one-half maximum inhibition (20 μM) is far higher than that required to block the cation-ATPases with phosphorylated intermediates found in animal cells, yeast, and *Neurospora* sp. (8, 13).

Although there is substantial evidence for a proton-translocating ATPase in *M. gallisepticum*, the possibility of a second ATPase that translocates Na^+ cannot be entirely ruled out. This question can only be resolved by purification and reconstitution of the ATPase(s). The successful solubilization of a stable, DCCD-sensitive ATPase activity reported in this investigation is thus a vital first step toward the resolution of this question.

TABLE 6. Stability of detergent-solubilized ATPase activity[a]

Detergent (%)	Sp act of extracts (nmol of P_i/min per mg)	Half-life of solubilized activity stored at 4°C (days)				
		Control	5 mM $MgCl_2$	Diluted extract 1 : 1	Mycoplasma lipid	Glycerol
Octylglucoside (0.88)	451	1.2	0.5	3.4	1.2	6.5
Zwittergent 3-12 (0.1)	688	3.2	0.5	4.8	3.3	>14[b]

[a] Thawed membranes (1 mg of protein per ml) were solubilized in the presence of 1 mM dithiothreitol–10 mM Tris (pH 7.4) with the detergent indicated for 30 min at 4°C. The supernatants were stored at 4°C under five conditions: no additions, the addition of 5 mM $MgCl_2$, 1 : 1 dilution with 7.5 mM NaCl, 1 mM dithiothreitol, 2.5 mM Tris (pH 7.4), 1.7 mg of water-dispersed *M. gallisepticum* lipid per nl, or 24% (wt/wt) glycerol. ATPase activities were assayed periodically. The specific activity of the initial detergent-free membrane preparation was 395 nmol/min per mg.

[b] The sample retained 100% of its activity after 14 days of storage at 4°C.

TABLE 7. Inhibition of ATPase of membrane-bound and solubilized activities by DCCD and ADP[a]

Prepr	Addition	Sp act (nmol/min per mg)	% Activity
Membrane	None	306.1	100.0
	20 μM DCCD	47.4	15.5
	5 mM ADP	155.5	50.8
Detergent-solubilized enzyme	None	563.4	100.0
	20 μM DCCD	110.6	19.6
	5 mM ADP	272.0	48.3

[a] Membranes were incubated at 1 mg of protein per ml with 0.1% (wt/vol) Zwittergent 3-12–10 mM Tris (pH 7.4)–1 mM dithiothreitol and processed as described in footnote *b* of Table 5. The ATPase activities of the detergent-free membrane and detergent-solubilized enzyme (supernatant) were compared with or without the addition of DCCD or ADP.

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