Characterization of a Membrane-Associated ATPase from *Pseudomonas aeruginosa*[1] (34380)

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A variety of physiological roles have been assigned to membrane-bound ATPases[2], such as inorganic cation transport, coupling in oxidative phosphorylation, contraction of mitochondrial membranes, reversible contraction of skeletal myofibrils and actomyosin, a contractile process in bacteriophages, and regulation of permeability of the external cell membrane to extracellular solutes in bacteria. Since we are interested in mechanisms of permeability of *Pseudomonas aeruginosa*, we undertook an investigation to characterize the cell membrane-bound ATPase of this microorganism. It was hoped that this investigation might yield results which would reveal a role for ATPase in permeation mechanisms. While the ATPase of *P. aeruginosa* has been characterized by this research, the true physiological role of this enzyme, described below, still remains to be elucidated. However, the purpose of this communication is to report our observations on the ATPase of *P. aeruginosa* in order to extend present information on bacterial ATPases.

Materials and Methods. Cultivation of organism. P. aeruginosa strain OSU 64 was cultivated at 37° on a rotary shaker in a glucose–basal salts medium enriched with nutrient broth and yeast extract as previously described (1). The cells were harvested after 12 hr of cultivation and washed with 0.15 *M* NaCl before use.

Preparation of cell membranes. Washed cells collected from 1 liter of medium (approx 15 g of packed cells) were suspended in a 4:6 (v/v) ratio in cold (0–4°) 0.03 *M* Tris–HCl buffer, pH 7.5, to which was added $MgCl_2$ to give a final concentration of 0.03 *M* $MgCl_2$. The cells were ruptured with a French pressure cell at 16,000 lb/in^2 and the cell envelopes were collected by centrifuging at 0–4° for 1 hr at 23,500*g*. In order to remove cell wall components, the cell envelopes were incubated at 25° for 10 min in 150 ml of a system containing 33 μmoles of Tris–HCl buffer, pH 8; 1 μmole of EDTA, pH 8; and 30 μg of lysozyme/ml. The $MgCl_2$ was then added to the system to give a final concentration of 2 μmoles/ml. The cell membranes were collected by centrifuging at 0–4° for 1 hr at 23,500*g*. The cell membranes were further purified by incubating at 37° for

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[2] Abbreviations and trivial names. ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; CMB, *p*-chloromercuribenzoate; CTP, cytidine 5′-triphosphate; DNA, deoxyribonucleic acid; DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetate; fructose 6-*P*, fructose 6-phosphate; glucose 6-*P*, glucose 6-phosphate; GTP, guanosine 5′-triphosphate; IAA, iodoacetic acid; ITP, inosine 5′-triphosphate; ME, 2-mercaptoethanol; ATP, adenosine 5′-triphosphate (disodium salt); NADH, reduced nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; NEM, *N*-ethylmaleimide; PEP, phosphoenolpyruvic acid; Pi, inorganic phosphate; PP_i, inorganic pyrophosphate; RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; TTP, thymidine 5′-triphosphate; UTP, uridine 5′-triphosphate; ATPase, ATP phosphohydrolase (EC 3.6.1.3); DNase, deoxyribonucleate-3′-nucleotidohydrolase (EC 3.1.4.6); glucose 6-phosphate dehydrogenase, D-glucose-6-phosphate:NADP oxidoreductase (EC 1.1.1.49); hexokinase, ATP:D-hexose 6-phosphotransferase (EC 2.7.1.1.); lactate dehydrogenase, L-lactate:NAD oxidoreductase; lysozyme, mucopeptide *N*-acetylmuramylhydrolase (EC 3.2.1.17); pyruvate kinase, ATP:pyruvate phosphotransferase (EC 2.7.1.40); trypsin (EC 3.4.4.4.); RNase, ribonucleate nucleotide-2′-transferase (cyclizing) (EC 2.7.7.17).

30 min in 30 ml of a system containing 30 μmoles of Tris–HCl buffer, pH 7.5; 20 μg of DNase; and 20 μg of RNase/ml. The cell membranes were collected by centrifuging at 0–4° for 1 hr at 23,500*g*, then washed once in 50 ml of cold (0–4°) 0.03 *M* Tris–HCl buffer, pH 7.5, and collected by centrifuging at 23,500*g* for 1 hr. The washed cell membranes were next suspended in 25 ml of 0.001 *M* Tris–HCl buffer, pH 7.5, and dialyzed against several changes of the same buffer for 48 hr at 0–4°.

Cell membranes prepared in this manner contained no RNA when assayed by the orcinol technique (2). Less than 10 μg of DNA/milligram of cell membrane protein was detected when DNA was assayed by the diphenylamine technique (2). Protein was estimated by the biuret method (3). No attempts were made to determine contamination of the cell membranes by cell wall components.

The cell membranes could be frozen and thawed several times with no loss of ATPase activity. Moreover, we have stored these cell membrane preparations of 0° in an ice bath for as long as 2 weeks without noting a loss of ATPase activity. On the other hand, if the final dialysis step was omitted during the isolation and purification procedures, the cell membranes were labile to freezing but stable for several days at 0° when stored in an ice bath.

Assay of ATPase activity. Unless otherwise indicated, ATPase activity was determined by measuring the liberation of P_i in a test system that contained 10 μmoles of ATP; 1 μmole of $MgCl_2$; 100 μmoles of Tris–acetate buffer, pH 9; and 0.1 mg of cell membrane protein in a final volume of 1 ml. This system was incubated for 30 min at 37°. The reaction was stopped by the addition of 0.2 ml of 10% trichloracetic acid. Precipitated protein was removed by centrifuging for 5 min at 8000*g*. Inorganic phosphate was estimated by the Fiske and Subbarow technique as modified by Lohmann and Jendrassik (4). Corrections were made for enzyme and reagent blanks. Variations in the amount or type of reactants and in additions of other reagents to this system are described in the text as appropriate. Specific activity is defined as μmoles of P_i liberated $\times$ min^{-1} $\times$ (mg of protein)$^{-1}$.

Assay of end products of ATPase activity. The reaction for ATPase activity was terminated by boiling after 30 min of incubation. The denatured protein was sedimented by centrifuging and discarded. Residual ATP in 0.1 ml of the supernatant was estimated by adding 10 μmoles of glucose; 2 units of glucose 6-phosphate dehydrogenase; 2 units of hexokinase; 75 μmoles of $MgSO_4$; 100 μmoles of phosphate buffer, pH 7.4; and 1 μmole of NADP in a total volume of 3 ml. The reaction was incubated at 25° and the reduction of NADP was measured at 340 nm.

The quantity of ADP in 0.1 ml of the supernatant was measured by adding 10 μmoles of phosphoenolpyruvate; 300 μmoles of Tris–HCl buffer, pH 7.5; 75 μmoles of $MgSO_4$; 75 μmoles of KCl; 1 μmole of NADH; 2 units of pyruvate kinase; and 2 units of lactate dehydrogenase in a total volume of 3 ml. The reaction was incubated at 25° and the oxidation of NADH was measured at 340 nm.

The P_i was estimated as described for the assay of ATPase activity.

Biological reagents and enzymes. All biological reagents (*e.g.*, cofactors and enzymes) used for the assays described herein were obtained in the highest state of purity available from the Sigma Chemical Co.

Results. Effect of pH and divalent cations. Preliminary experiments established that maximal ATPase activity occurred in the presence of Mg^{2+} at an alkaline pH. Maximal activity was obtained at pH 9 (Fig. 1) and the enzyme was maximally activated by 1 m*M* Mg^{2+} (Fig. 2). The ATPase also showed a broad requirement for divalent cations. While Mg^{2+} was the better activator, the ATPase was also activated strongly by Mn^{2+}, Zn^{2+}, and Ca^{2+} and weakly activated by Cu^{2+} and Ni^2 (Fig. 2). Maximal activity was observed with a final concentration of 1 m*M* Mg^{2+} in the presence of 10 m*M* ATP at pH 9 (Fig. 2). Increased concentrations of Mg^{2+} depressed or inhibited the activity. On the other hand, maximal activation of the ATPase by the other

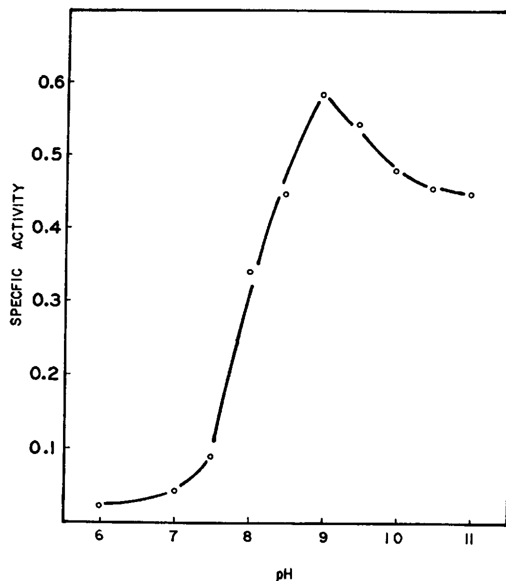

FIG. 1. The effect of pH on ATPase activity in assay systems containing 100 μmoles of Tris–acetate buffer (pH as indicated), 10 μmoles of ATP, 1 μmole of $MgCl_2$ and 0.1 mg of cell membrane protein in a final volume of 1 ml.

divalent cations required concentrations higher than Mg^{2+}. The ATPase was also inhibited by increased concentrations of these divalent cations. Although not shown here, addition of EDTA partially reversed the inhibition by increased concentrations of divalent cations.

Under these conditions (*i.e.*, pH 9, 1 m*M* Mg^{2+}, 10 m*M* ATP, and 0.1 mg of cell membrane protein/ml), there was a curvilinear production of P_i throughout an incubation period in excess of 120 min. When an incubation period of 30 min was used, a reproducible specific activity of 0.64±0.02 was observed with the various cell membrane preparations. The maximal rate of hydrolysis of ATP was achieved when a concentration of 8–9 m*M* ATP was used and increased concentrations of ATP did not depress the rate up to a concentration of 20 m*M* ATP, the highest concentration used in these experiments. The ATPase had an approximate K_m of 3 m*M*.

When assays were made for end products of ATPase activity, only ADP and P_i were detected. For each μmole of ATP hydrolyzed, one μmole each of ADP and P_i were produced. Actual values for one experiment were as follows: 1.9 μmoles of ATP were hydrolyzed and 2.0 μmoles of P_i and 1.8 μmoles of ADP were detected.

Absence of stimulation by Na^+ and K^+. The alkali cations, Na^+ and K^+, had no stimulating effect at low concentrations on the ATPase activity when added to the assay system but high concentrations of these cations depressed activity. Specifically, 10 m*M* Na^+ or K^+ had no effect on ATPase activity while concentrations of 40 and 120 m*M* depressed activity by 10–12% and by 25–35%, respectively. Similarly, when Tris–ATP was used as substrate instead of the disodium salt of ATP, neither Na^+ nor K^+, singly or in combination, stimulated activity.

Inhibitor studies. Of the various inhibitors tried, the ATPase was strongly inhibited by NaN_3, ADP and the sulfhydryl-binding agent, CMB, and it was weakly inhibited by AMP, KF, and NEM (Table 1.) The latter agent is also considered to be a sulfhydryl-

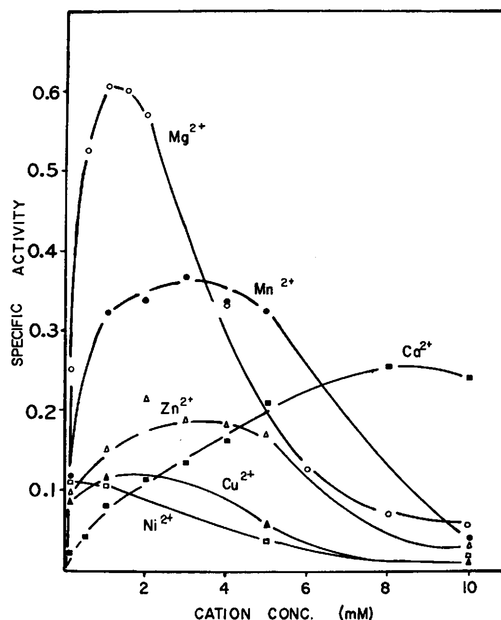

FIG. 2. The effect of various divalent cations as activators of ATPase in assay systems containing 100 μmoles of Tris–acetate buffer, pH 9, 10 μmoles of ATP, 0.1 mg of cell membrane protein, and divalent cations as indicated in a final volume of 1 ml.

TABLE I. The Effect of Various Inhibitors on ATPase Activity.

Inhibitor	(μmoles ml^{-1})	Inhibition (%)
NaN_3	1.0	96
CMB	0.1; 1.0	56; 82
NEM	5.0	11
ADP	5.0	62
AMP	5.0	14
KF	5.0	8
KCN	10.0	0
Atabrine	0.1	0
DNP	0.5	0
IAA	10.0	0
Ouabain	5.0	0
Oligomycin	100.0[a]	0

[a] Concentration (μg ml^{-1}).

binding agent. The inhibition caused by CMB and NEM was fully reversed by ME.

We were unable to discern from these experiments the nature of the inhibitory effects of ADP, *i.e.,* whether competitive or noncompetitive inhibition was involved. In relation to concentration, sigmoidal types of curves resulted.

Substrate specificity. The ATPase had greatest substrate activity for ATP. It was also active against the other purine nucleotides, GTP and ITP (Table II). The activity for GTP and ITP, however, was only about 30% as great as that for ATP.

It could not be discerned from these experiments whether the low level of activity observed for the pyrimidine nucleotides, TTP and UTP, was actual or due to contaminating purine nucleotides. No activity, however, was observed for CTP.

TABLE II. Substrate Specificity of ATPase.

Substrate[a]	Activity (%)
ATP	100
ITP	30
GTP	29
UTP	5
TTP	4
CTP	0
ADP	0
PP_i	0
Glucose 6-*P*	0
Fructose 6-*P*	0

[a] Substrate concentration (10 μmoles ml^{-1}).

No phosphatase activity was observed by the cell membrane preparation for ADP, glucose 6-*P* or fructose 6-*P* and no pyrophosphatase activity was noted for PP_i (Table II).

Discussion. We elected to characterize only the membrane-bound ATPase of *P. aeruginosa* and no attempt was made to study this enzyme in a solubilized or purified state. The ATPase of *Streptococcus faecalis* (5) and of *Micrococcus lysodeikticus* (6) showed different properties in the soluble and membrane-bound states, a property defined as "allotopy" (7). Thus, we assumed that the true properties of the ATPase of *P. aeruginosa* would be those of the membrane-bound enzyme.

The most definitive studies reported to date on bacterial ATPases have been done with gram-positive anaerobic or facultative anerobic bacteria (5, 6, 8). Since *P. aeruginosa* is a gram-negative obligate aerobe, we also wished to compare our results to those reported by others.

The ATPase of *P. aeruginosa* was maximally activated by Mg^{2+} at pH 9. Other divalent cations, especially Mn^{2+}, Ca^{2+}, and Zn^{2+}, also activated the ATPase but they were less effective activators than Mg^{2+} since higher concentrations were required and lower specific activities resulted. Under our experimental conditions, maximal activation resulted when Mg^{2+} and ATP were in a 1:10 ratio, respectively. The ATPases from other bacteria have also been reported to require Mg^{2+} but a 1:1 or a 1:2 ratio of Mg^{2+} and ATP was reported to be required for maximal activity (6, 9–19). The ATPase of *P. aeruginosa* may differ in this respect from ATPases of other bacteria. However, we did not determine how much Mg^{2+} was naturally bound to the cell membranes. Thus, the Mg^{2+} available to the enzyme may have been in excess to that which we added to the assay systems. When the concentration of Mg^{2+} or of other divalent cations was increased beyond the amount required for optimal activation, the ATPase activity was depressed. Excess Mg^{2+} has also been reported to be inhibitory toward the ATPase of *S. fecalis* (9).

It was reported recently that *M. lysodeikticus* possesses a Ca^{2+}-dependent ATPase (6). However, this property was demonstrated by purified enzyme. The membrane-bound ATPase was maximally activated by Mg^{2+}.

Na^+ and K^+ did not stimulate the ATPase of *P. aeruginosa*. Similarly, Na^+ and K^+ were reported to have no effect on the ATPases of *M. lysodeikticus* (6), *Mycoplasma laidlawii* (16), *Lactobacillus arabinosus* (20) or *Vitreoscilla* (10). On the other hand, the ATPases of *Myxococcus xanthus* (17), a marine species of *Pseudomonas* and of *Cytophaga* (11), *Staphylococcus aureus* (12), *L. fermenti* (15), and *Macillus megaterium* (21) were reported to be further stimulated by the addition of Na^+ or K^+ to the assay system. In the latter cases, the stimulation by Na^+ or K^+ may have been due to the formation of an optimal ionic strength of the assay system rather than to any unique requirement for Na^+ or K^+.

The ATPase of *P. aeruginosa*, therefore, resembles ATPases from other bacteria by the requirement for Mg^{2+} and for an alkaline pH and by the absence of stimulation by Na^+ and K^+. It also shares these characteristics with the ATPase from the inner membrane of beef heart mitochondria (22). Specific activity and K_m values also agree closely (6, 23, 25).

While the ATPase of *P. aeruginosa* had highest activity against ATP, the purine nucleotides, GTP and ITP, were also hydrolyzed at about 30% of the rate of ATP hydrolysis. Little or no activity was observed against the pyrimidine nucleotides. No phosphatase or pyrophosphatase activity was detected. Moreover, when ATP was used as substrate, ADP and P_i were the sole end products. The ATPases from other bacteria have also been reported to be active against nucleoside triphosphates other than ATP and to have no phosphatase activity (8–12, 16, 20).

No ATPase activity by intact cells was detected. Thus, we conclude either that the ATPase of *P. aeruginosa* is bound to the interior surface of the cell membrane or that it has an intramembrane location. The ATPase of *M. xanthus* has also been reported to be located within the cell membrane (17). Similarly, the membrane–ATPase complex of *M. lysodeikticus* shows no hydrolytic activity for ATP until the system is activated by trypsin (6).

The membrane-bound ATPase of *P. aeruginosa* was stable to freezing after the cell membranes were dialyzed against Tris buffer. Before dialysis, the ATPase activity was lost when the cell membranes were frozen. These results indicated that a low molecular weight, dialyzable substance induced the cold inactivation of the ATPase of *P. aeruginosa*. Similarly, Abrams (23) reported that ADP or Mg^{2+}–ATP induced inactivation of the ATPase of *S. faecalis* by the cold. Whether the cold inactivation of the ATPase of *P. aeruginosa* was due to the presence of ADP or of Mg2+ ATP in the undilyzed cell membrane preparations or due to some other substance could not be discerned from these experiments.

Inhibitors were used to further characterize the ATPase of *P. aeruginosa*. It was not inhibited by ouabain, oligomycin, atabrine, DNP, or KCN. It was strongly inhibited by NaN_3 which may be due to the metal-binding properties of this inhibitor. Of the sulfhydryl-binding agents that were used, the ATPase was strongly inhibited by CMB, weakly inhibited by NEM and not inhibited by IAA. The inhibition by CMB and NEM was fully reversed by ME. We interpret these results to indicate that the active sulfhydryl group(s) of the ATPase were accessible to the planar CMB molecule but inaccessible to NEM and IAA.

The ATPase of *P. aeruginosa* was strongly inhibited by ADP. ADP has also been reported to inhibit the ATPases of *M. lysodeikticus* (6), *B. megaterium* (21), and *S. faecalis* (23). In the latter case, the inhibition was reported to be competitive. Our results, however, are in agreement with those of Muñoz *et al.* (6) who reported that the inhibitory effects of ADP on the ATPase of *M. lysodeikticus* in relation to concentration gave sigmoidal curves, suggesting an allosteric enzyme and a regulatory property for

ADP. Moreover, these results support the conclusions drawn by Muñoz *et al.* (6) and by Schnebli and Abrams (24) that the ATPases of *M. lysodeikticus* and *S. faecalis* are composed of subunits. Thus, allosteric properties would be expected.

There was no correlation between factors (*e.g.*, divalent cations and pH) previously reported to affect the transport of substrates by *P. aeruginosa* (1) and factors affecting ATPase activity. From these observations, then, we were unable to relate ATPase activity to transport mechanisms. Similarly, we were not able to discern the true physiological role of the membrane-bound ATPase of *P. aeruginosa*. However, our results, when compared to reports by other investigators, indicate that all bacterial ATPases have very similar properties irrespective of the physiological type of bacterial cell used as the source of the enzyme. Similarly, bacterial ATPases appear to share many properties with mitochondrial ATPases.

Summary. The cell membrane-bound ATPase of *P. aeruginosa* was characterized. It was optimally activated by Mg^{2+} and suboptimally activated by other divalent cations (*e.g.*, Mn^{2+}, Ca^{2+}, or Zn^{2+}) at pH 9. It was not stimulated by Na^+ or K^+. Concentrations of divalent cations higher than those required for optimal activation of the ATPase were inhibitory. The ATPase had greatest activity against ATP. Other purine nucleotide triphosphates were hydrolyzed by the ATPase at 30% of the rate of ATP. The ATPase had little or no activity against the pyrimidine nucleoside triphosphates or against ADP. The products of ATP hydrolysis were ADP and inorganic phosphate. No phosphatase or pyrophosphatase activity was detected. The ATPase was not inhibited by ouabain, oligomycin, iodoacetic acid, 2,4-dinitrophenol, Atabrine or KCN. It was strongly inhibited by NaN^3, *p*-chloromercuribenzoic acid (CMB) and ADP and it was weakly inhibited by *N*-ethylmaleimide (NEM), AMP, and KF. The inhibition by CMB and NEM was reversed by 2-mercaptoethanol. The ATPase appeared to be located on the inner surface of the cell membrane or to have an intramembrane location. The true physiological role for the ATPase was not elucidated by this research.

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