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Identification of a Vanadate-Sensitive, Membrane-Bound ATPase in the Archaebacterium *Methanococcus voltae*

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Membrane-bound ATPase activity was detected in the methanogen *Methanococcus voltae*. The ATPase was inhibited by vanadate, a characteristic inhibitor of E_1E_2 ATPases. The enzyme activity was also inhibited by diethylstilbestrol. However, it was insensitive to *N,N'*-dicyclohexylcarbodiimide, ouabain, and oligomycin. The enzyme displayed a high preference for ATP as substrate, was dependent on Mg^{2+}, and had a pH optimum of approximately 7.5. The enzyme was completely solubilized with 2% Triton X-100. The enzyme was insensitive to oxygen and was stabilized by ATP. There was no homology with the *Escherichia coli* F_0F_1 ATPase at the level of DNA and protein. The membrane-bound *M. voltae* ATPase showed properties similar to those of E_1E_2 ATPases.

Methanococcus voltae is a heterotrophic methanogen which uses hydrogen and carbon dioxide or formate as an energy source (37). Although many methanococci grow autotrophically in mineral medium, *M. voltae* requires acetate, leucine, and isoleucine for growth in mineral medium (37). This organism also carries out energy-dependent uptake of amino acids, and there is evidence that such transport is mediated by a transmembrane sodium gradient (21). Recently, the presence of an energy-dependent, carrier-mediated uptake system for HS-coenzyme M and CH_3-S-coenzyme M has been suggested (32).

The mechanism of coupling between methane formation and ATP synthesis in methanogens has been a topic of controversy. The concomitant decrease in ATP formation and membrane potential caused by the addition of protonophores to *Methanosarcina barkeri* has led to the conclusion that ATP synthesis is driven by a chemiosmotic gradient of protons generated by the reactions of methanogenesis (3). An earlier report that methanogenesis from H_2 and CO_2 as well as ATP synthesis can proceed in *Methanobacterium thermoautotrophicum* in the absence of a measurable membrane potential (33) has recently been clarified in studies with protoplasts of this methanogen. It has been shown that ATP synthesis, methanogenesis, and the membrane potential decrease in parallel in protoplasts treated with the protonophore 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF-6847) (27). The inability of the ionophore to reach the internal membranes in whole cells has been proposed to explain this difference in sensitivity. On the basis of evidence that electron transfer-driven ATP synthesis in *Methanococcus voltae* is not dependent on a proton electrochemical gradient, a molecular scheme in which ATP synthesis is coupled directly to electron transfer has been proposed (10, 25). Furthermore, the *M. voltae* ATPase is not considered to function physiologically as an ATP synthase; rather, it is believed to be involved in electrogenic sodium translocation (1, 8).

ATP-driven ion pumps fall into two classes (26). The F_0F_1 ATPases are structurally complex. They translocate protons and are inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD). They can carry out both ATP synthesis and ATP hydrolysis. The F_0F_1 ATPases characterized for bacteria, mitochondria, and chloroplasts display similar structural properties and a significant conservation of amino acid sequence (11). The characterization of an ATPase as an E_1E_2 type depends on either of two criteria: (i) inhibition of activity by vanadate or (ii) formation of an acylphosphate intermediate during ATP hydrolysis, with the phosphate binding to an aspartate residue (M. O. Walderhaug, G. Saccomani, T. H. Wilson, D. Briskin, R. T. Leonard, G. Sachs, and R. L. Post, Fed. Proc. **42**:1275, 1983). The E_1E_2 ATPases function physiologically in the direction of ATP hydrolysis, vary with respect to cation specificity (depending on the system), and usually have a simple structure, often being composed of a single polypeptide chain.

In this paper we demonstrate the presence in *M. voltae* of a vanadate-sensitive, DCCD-resistant, membrane-associated ATPase. This is the first report of an ATPase with these properties in archaebacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. voltae* PS (DMS1537) was grown in a defined medium (37) supplemented with 0.05% yeast extract in a pressurized atmosphere of H_2-CO_2 (80 and 20% [vol/vol], respectively) at 32°C in 1-liter Wheaton bottles (1) containing 200 ml of growth medium. *Escherichia coli* W3110 was grown as described by Vogel and Steinhart (36).

Preparation of membranes. *M. voltae* cells were grown to late log phase (optical density at 660 nm, 0.6 to 0.8 with approximately 0.15 mg of cell protein per ml) and harvested aerobically by centrifugation at 4°C for 15 min at 5,000 × *g*. The harvested cells were suspended aerobically in twice the cell pellet volume of a mixture of 50 mM glycine–NaOH (pH 7.5)–50 mM KCl–5 mM $MgCl_2$ (G buffer), 1 mM ATP, and 10% glycerol. DNase (10 μg/ml) was added, and the cell suspension was passed through a French pressure cell at 110 MPa. The cell debris was removed by centrifugation at 4°C for 10 min at 10,000 × *g*. The supernatant was centrifuged at 4°C for 2 h at 100,000 × *g*, and the resulting pellet was washed once with and suspended in G buffer. The resulting membrane-containing fraction (15 to 20 mg of protein per ml) was loaded on a 30 to 60% (wt/wt in H_2O) sucrose gradient and centrifuged at 20°C for 16 h at 55,000 × *g* in a Beckman

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SW28 rotor. For inhibitor studies, the membrane fractions from the sucrose gradient were pooled and the sucrose was removed by washing with G buffer. For some experiments, membranes were prepared in an identical manner but with all steps carried out under anaerobic conditions. *E. coli* membranes were prepared as described by Vogel and Steinhart (36).

Enzyme assays. ATPase activity was measured in G buffer by the release of inorganic phosphate (9) or by the disappearance of ATP by the luciferin-luciferase method (23) with the Turner Designs model 20E Luminometer. A typical reaction mixture had a volume of 0.5 ml and contained 0.1 to 0.3 mg of protein. The reaction was started by the addition of 5 mM ATP and followed for 20 min at 37°C. All determinations were performed under protein-limiting conditions.

Protein determination. Protein was assayed by the Bradford method (5) with bovine serum albumin as a standard. The amount of protein present in the fractions from the sucrose gradients was estimated by absorbance at 280 nm.

Western blots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done with the Laemmli (24) buffer system. *E. coli* membranes were used as positive controls. Membranes were treated with sodium dodecyl sulfate (final concentration, 3.2%) and heated at 60°C for 30 min before being applied to the gel. Western blots (immunoblots) were performed as described elsewhere (6). After the proteins were transferred, the nitrocellulose was washed at room temperature as follows. The first wash was performed for 1.5 h in 10% fetal calf serum and phosphate-buffered saline (PBS) containing 10 mM sodium phosphate with 0.15 M sodium chloride. Next, immunoglobulin G (10 mg) raised against F_1 ATPase (provided by R. Simoni, Stanford University, Palo Alto, Calif.) was added to the 10% fetal calf serum–PBS, and the mixture was incubated for 3 h. The incubation was followed by two 20-min washes with PBS-Triton X-100 (0.05%) and a 10-min wash with PBS alone. The filter was then incubated for 20 min with 10% fetal calf serum–PBS which contained ^{125}I-protein A (5 μl contained 4 × 10^6 cpm). The filter was washed once again with PBS, PBS–Triton X-100, and PBS, as described above. It was then air dried and autoradiographed for 1 to 7 days at −70°C.

Southern blots. DNA from *M. voltae* and *E. coli* was extracted as described by Saito and Miura (31) except that lysozyme was not used and proteinase K was used instead of pronase during the extraction of *M. voltae* DNA. pRPG54 (15), a plasmid which has the entire *E. coli* ATPase operon, was labeled by nick translation with [α-^{32}P]dATP (29). *M. voltae* DNA was digested to completion with restriction enzyme *Hin*dIII, *Cla*I, *Eco*RI, or *Pst*I, electrophoresed on 1% agarose gels, and then transferred to nitrocellulose, as described by Southern (34). The filter was first incubated in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% Denhardt solution–100 μg of salmon sperm DNA per ml for 2 to 3 h at room temperature. The incubation was followed by hybridization with denatured ^{32}P-labeled pRPG54 DNA in 6× SSC–0.01 M EDTA–5× Denhardt solution–0.05% sodium dodecyl sulfate–100 μg of denatured salmon sperm DNA per ml at room temperature for 14 h. The stringencies of subsequent washes were varied by changing the temperature and salt conditions. These were carried out at 25, 37, and 50°C. The salt conditions used were 2×, 1×, and 0.1× SSC.

Staining for enzyme activity. ATPase activity was located on nondenaturing 10% polyacrylamide gels (28). The gel was incubated with 50 mM glycine–NaOH buffer (pH 7.5) containing 5 mM ATP, 5 mM $MgCl_2$, and 0.05% lead acetate for 40 min. It was subsequently developed in 0.1% sodium sulfide.

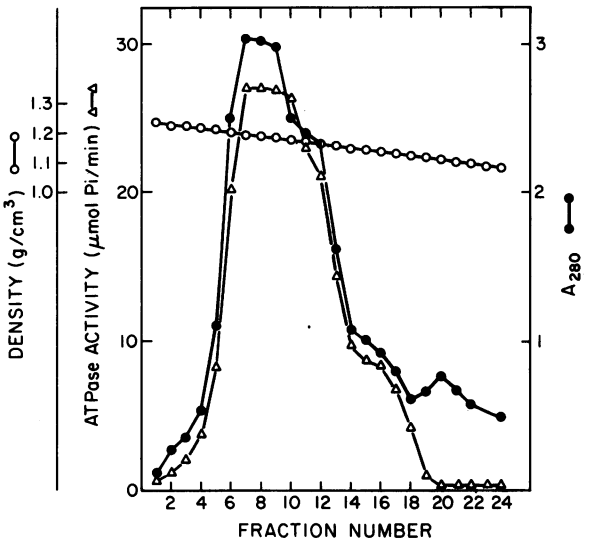

FIG. 1. Sucrose gradient centrifugation profile of the washed 100,000 × *g* membrane pellet.

Materials. Sodium orthovanadate, oligomycin, ouabain, diethylstilbestrol, and DCCD were purchased from Sigma Chemical Co. The luciferin-luciferase enzyme system was purchased from Turner Designs.

RESULTS

Characterization of ATPase activity. When *M. voltae* cells were broken in a French press and then subjected to centrifugation at 100,000 × *g*, 95% of the ATPase activity originally present in the crude cell extract was recovered in the pellet fraction. Sucrose gradient analysis of this pellet fraction yielded a major fraction of 280-nm-absorbing material whose density ranged from 1.16 to 1.20 g/cm^3 (Fig. 1). Since the profile of incorporated mevalonic acid, a marker for membrane lipid (35), coincides with this material, it was apparent that the material represented membranes (N. Santoro and J. Konisky, unpublished experiments). As can be seen in Fig. 1, ATPase activity was localized to this fraction, which indicated that it was a membrane-associated enzyme.

The addition of 2% Triton X-100 to the membrane fraction resulted in complete solubilization of the ATPase (Fig. 2) and in an increase in the specific activity of the enzyme from 14.3 to 21.1 μmol/min per mg of protein. In the absence of detergent, G buffer containing 500 mM KCl solubilized 45% of the enzyme activity. The significance of this result has not been investigated further.

It was possible to specifically stain for ATPase activity on nondenaturing polyacrylamide gels. In such an analysis (data not shown), a single band of activity was observed, although we did on occasion observe additional weaker bands. The weaker bands may have represented enzymatically active degradation products of the native enzyme, or perhaps minor ATPase species. When assayed by staining on nondenatured polyacrylamide gels, both the cell extract and the 100,000 × *g* pellet fraction contained ATPase, while the 100,000 × *g* supernatant did not. In contrast, the pellet fraction obtained after treatment of the membrane with 2%

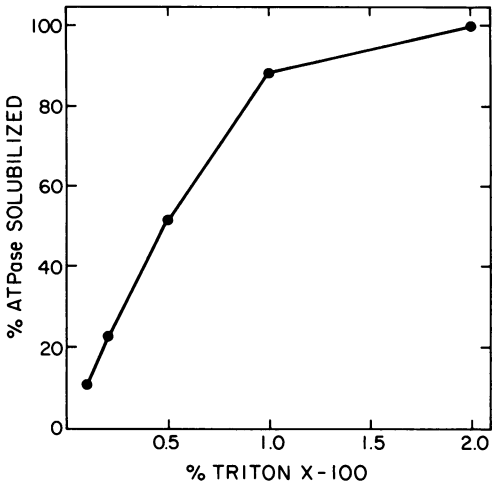

FIG. 2. Solubilization of *M. voltae* ATPase by Triton X-100. The 100,000 × *g* membrane pellet (protein concentration, 5 mg/ml) was treated with suspension buffer containing various amounts of Triton X-100 at 4°C for 30 min and centrifuged at 4°C for 2 h at 100,000 × *g* to obtain the soluble and membrane fraction.

Triton X-100 contained no assayable activity, while this procedure led to the solubilization of 100% of the ATPase activity. Furthermore, the washed membranes from fraction 8 of the sucrose gradient (Fig. 1) also contained ATPase. These results were consistent with the results of the sucrose gradient analysis and provided further evidence for the membrane association of this ATPase.

Properties of the membrane-associated enzyme. Washed membranes obtained from the sucrose gradients were used in these studies. ATP hydrolysis activity was optimum at pH 7.5 (Fig. 3) and required the presence of Mg^{2+}. Its absence from the assay mixture resulted in only 5% of the activity observed when 5 mM $MgCl_2$ was present. HSO_3^-, HCO_3^-, or SO_4^{2-} (25 mM) had no effect on ATPase activity. This finding was in contrast to results with *Methanosarcina barkeri* ATPase, which is stimulated by HSO_3^- (20).

The enzyme displayed a substrate preference for ATP (Table 1). Over the concentration range examined (2 to 10 mM), 5 mM ATP was optimal. When GTP and ITP were

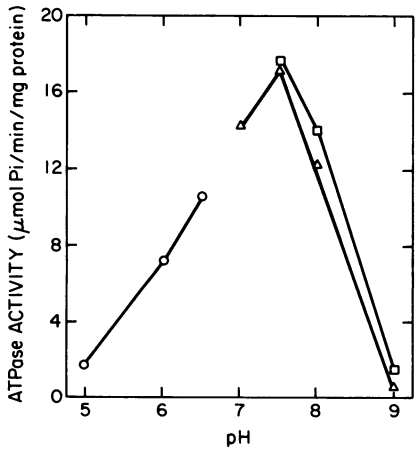

FIG. 3. Effect of pH on membrane-bound *M. voltae* ATPase activity. All buffers (0.1 M acetate [○], Tris [△], and glycine-NaOH [□]) contained 1 mM ATP, 5 mM $MgCl_2$, and 50 mM KCl.

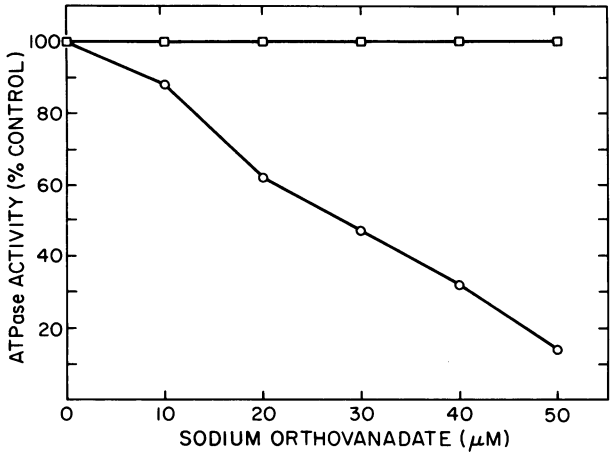

FIG. 4. Effect of sodium orthovanadate on ATPase activity. For *E. coli* ATPase (□), 100% activity represents 38 μmol of P_i/min per mg of protein. For *M. voltae* ATPase (○), 100% activity represents 16.5 μmol of P_i/min per mg of protein.

used as substrates, the enzyme activity was only 45 and 32%, respectively, as compared with that of ATP. UTP (15%) and TTP (7%) were poor substrates, and PP_i was not hydrolyzed, which ruled out the presence of a phosphatase activity. The ATPase activity was the same whether it was measured by P_i release or by decrease in ATP level.

We found that the inclusion of 1 mM ATP in buffer solutions greatly stabilized the ATPase. The membrane-bound enzyme was stable at 4°C in G buffer containing 1 mM ATP for at least 2 weeks. ATPase activity was not sensitive to the presence of oxygen, and we observed no difference in either enzyme level or inhibitor specificity in enzyme preparations made aerobically or anaerobically. Incubation of the membrane-bound enzyme for 10 min with trypsin (150 μg/ml) at 37°C resulted in complete loss of activity.

Sensitivity to vanadate and diethylstilbestrol. Several inhibitors of eubacterial and eucaryotic ATPases were tested for their effects on membrane-bound *M. voltae* ATPase. There was an 86% inhibition of *M. voltae* ATPase activity with 50 μM vanadate, an inhibitor of E_1E_2-type ATPases (Fig. 4). In contrast, 50 μM vanadate had no effect on the *E. coli* ATPase activity. Diethylstilbestrol, an inhibitor of vanadate-sensitive membrane ATPases of plants (2) and fungi (4), caused 92% inhibition of *M. voltae* ATPase activity at 150 μM (Fig. 5), while no effect was observed on *E. coli* ATPase activity. In contrast, DCCD, an inhibitor of F_0F_1 ATPases (13), had no effect on *M. voltae* ATPase activity up to a concentration of 100 μM (data not shown), but it completely abolished *E. coli* ATPase activity. Similarly, oligomycin

TABLE 1. Substrate specificity of *M. voltae* ATPase

Substrate (5 mM)	Sp act (μmol of P_i/min per mg of protein)	Relative activity (%)
ATP	16.0	100
GTP	7.17	45
ITP	5.15	32
UTP	2.34	15
TTP	1.17	7
CTP	0.93	6
Sodium PP_i	ND	ND

[a] ND, Not detected.

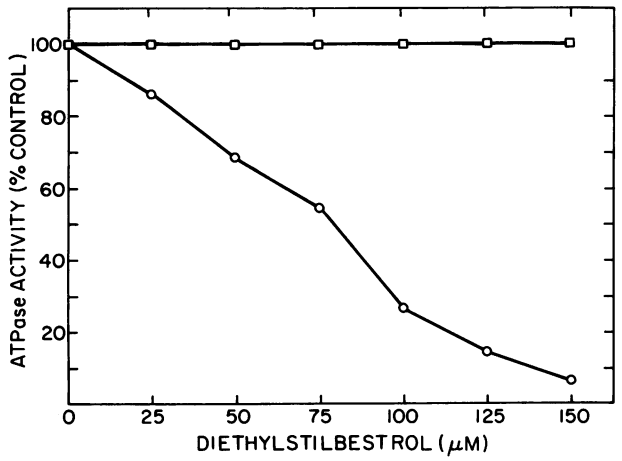

FIG. 5. Effect of diethylstilbestrol on ATPase activity. For *E. coli* ATPase (□), 100% activity represents 34 μmol of P_i/min per mg of protein. For *M. voltae* ATPase (○), 100% activity represents 18 μmol of P_i/min per mg of protein.

(10 μg/mg of protein), an inhibitor of the F_0 part of F_0F_1 ATPases of mitochondria and chloroplasts (13), had no effect on *M. voltae* and *E. coli* enzyme activity. The ATPase activities were also insensitive to the presence of ouabain (200 μM), an inhibitor of Na^+,K^+-ATPase (7). In all cases, identical results were obtained with the solubilized enzyme.

Lack of homology with *E. coli* or *Neurospora* ATPase. Homology between the F_0F_1 eubacterial and eucaryotic ATPases has been observed at the level of DNA and protein primary structure (11, 14). To investigate the homology at the level of DNA, ^{32}P-labeled plasmid pRPG54, which contains the entire *E. coli* ATPase operon, was used to probe total *M. voltae* DNA. The homology at the protein level was tested by probing *M. voltae* membrane proteins with immunoglobulin G raised against *E. coli* F_1 ATPase with ^{125}I-protein A. No cross-reaction was observed in either case. Furthermore, the addition of the *E. coli* antibody to reaction assay mixtures did not inhibit *M. voltae* ATPase activity. These experiments ruled out the possibility that the *M. voltae* ATPase shared significant homology with the *E. coli* F_0F_1 ATPase.

Since the *M. voltae* ATPase displayed a pattern of inhibition similar to that observed with the *Neurospora crassa* plasma membrane ATPase (4), we examined the possibility that the two enzymes shared sufficient structural homology to be detectable by Western blot analysis, with the antibody raised against the fungal enzyme as the probe. No cross-reactivity was observed (R. M. Dharmavaram and K. Allen, unpublished observation).

DISCUSSION

On the basis of the sensitivity of *M. voltae* to diethylstilbestrol and vanadate and its resistance to DCCD, we suggest that the *M. voltae* ATPase is not of the F_0F_1 type and may be more closely related to E_1E_2 ATPases. In contrast, the ATPase of *Methanosarcina barkeri* is sensitive to DCCD and has other features which suggest that it is related to F_0F_1 proton-translocating ATPases (20). Membrane-associated, DCCD-sensitive ATPase activity has been reported in the thermophilic *Methanobacterium thermoautotrophicum* (12). This enzyme, which is insensitive to vanadate (30), is likely of the F_0F_1 type. These results raise the possibility that the mechanism of ATP formation in *M. voltae* may differ significantly from that utilized by these other methanogens.

There is evidence that the physiological function of the *M. voltae* ATPase is Na^+ translocation (8). This would provide a mechanism for the direct coupling of ATP hydrolysis to the generation of the sodium motive force which supports active transport of several amino acids in this organism (21). Several E_1E_2 ATPases have been shown to mediate ATP-dependent translocation of cations other than protons (26).

Since ATP synthesis in *M. voltae* is not diminished under conditions in which the transmembrane electrical potential is collapsed by the addition of the protononophore SF6847, substrate-level phosphorylation has been proposed as the physiological mechanism of ATP synthesis (10, 25). Such a mechanism would obviate the need for a reversible proton-translocating ATPase in this organism. The 80 to 90% inhibition of the *M. voltae* ATPase by vanadate or diethylstilbestrol and the total resistance of the enzyme to DCCD which we observed are consistent with the absence of an F_0F_1 ATPase in this methanogen. Nevertheless, we cannot at this time completely exclude the possibility that this methanogen contains minor levels of an ATPase of this type.

The most thoroughly studied E_1E_2 ATPases have been those of the plasma membranes of fungal, plant, and animal cells. Although reports of E_1E_2 ATPases in procaryotes have been limited to *Streptococcus faecalis* (17, 19), *E. coli* (18), and *Acholeplasma laidlawii* (22), it is likely that their distribution is more widespread. The presence of an E_1E_2 ATPase in *M. voltae* extends its distribution to the third biological kingdom (38). Comparison of the amino acid sequence of the *E. coli* K^+-ATPase with that of the Ca^{2+}-ATPase of the sarcoplasmic reticulum indicates significant sequence homology (18). The K^+-ATPase of *E. coli* displays a smaller, though still significant, degree of homology to the plasma membrane E_1E_2 ATPase of *N. crassa*, which itself manifests significant homology with Na^+-, K^+-, and Ca^{2+}-transporting ATPases of animal cells (16). Whether such homology reflects convergent or divergent evolution is impossible to assess.

Future characterization of the *M. voltae* enzyme and its structural gene will provide a third perspective on the utility of these enzymes and may lead to insights into the evolution of mechanisms of ion translocation across membranes.

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