Characterization of a Membrane-Associated ATPase from *Methanococcus voltae*, a Methanogenic Member of the Archaea

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A membrane-associated ATPase with an M_r of approximately 510,000 and containing subunits with M_r s of 80,000 (α), 55,000 (β), and 25,000 (γ) was isolated from the methanogen *Methanococcus voltae*. Enzymatic activity was not affected by vanadate or azide, inhibitors of P- and F_1 -ATPase, respectively, but was inhibited by nitrate and bafilomycin A1, inhibitors of V_1 -type ATPases. Since dicyclohexylcarbodiimide inhibited the enzyme when it was present in membranes but not after the ATPase was solubilized, we suggest the presence of membrane-associated component analogous to the F_0 and V_0 components of both F-type and V-type ATPases. N-terminal amino acid sequence analysis of the α subunit showed a higher similarity to ATPases of the V-type family than to those of the F-type family.

A topic of recent research activity has been the delineation of the mechanism by which methanogenic bacteria couple the reduction of substrates to methane to produce ATP. These studies are of interest not only because these prokaryotes are members of the Archaea (36) and, thus, show characteristics of gene expression (7) and cell envelope structure (23) that distinguish them from the Bacteria but also because methanogens harbor a unique collection of electron carriers (13).

It has been shown that ATP production in methanogens is mediated by transmembrane chemiosmotic gradients, and there is evidence that the coupling ions may be either protons or sodium ions, depending on the methanogen. Investigations utilizing inverted vesicles of *Methanosarcina* strain Gö1 have shown that reduction of the heterodisulfide of coenzyme M (2-mercaptoethanesulfonate) and 7-mercaptoheptanoylthreonine phosphate leads to a proton motive force that drives ATP synthesis via an H^+ -translocating ATPase (for a review, see reference 3). In contrast, it has been proposed that ATP production in *Methanococcus voltae* is mediated by a sodium ion-translocating ATPase whose function is coupled to a sodium motive force that is generated through a primary sodium ion pump (15).

While it has been established that the membrane of *M. voltae* contains a component that is immunologically related to the H^+ -translocating ATP synthase of another member of the Archaea, *Sulfolobus acidocaldarius* (15), to date the *M. voltae* enzyme has not been isolated. Characterization of this enzyme will be important as we further define the underlying biochemical basis for ATP production in this marine methanogen. This paper reports the purification and partial characterization of a membrane-associated ATPase of *M. voltae* that we suggest is the ATP synthase.

MATERIALS AND METHODS

Growth, membrane preparation, and enzyme solubilization. *M. voltae* PS (DMS1537) was grown to an optical density at 660 nm of 0.6 to 0.8 in the defined medium of Whitman et al. (35) supplemented with 0.05% yeast extract

in a pressurized atmosphere of H_2 - CO_2 (80:20), pH 7.0, at 30°C and harvested by membrane-based tangential flow filtration (Millipore). The cell paste was suspended in 1,000 ml of buffer A (50 mM Tris-Cl [pH 7.5], 5 mM $MgCl_2$, 10% glycerol), and the cell suspension was flushed by the addition of hydrogen. When stored at 4°C for several weeks under an atmosphere of nitrogen, this suspension suffered no loss of ATPase activity, as assayed in cell extracts. Except when noted, all subsequent steps were carried out at 4°C under aerobic conditions. The cell suspension (200 ml, about 10 mg of cell protein per ml) was passed through a chilled French press cell at 10,000 lb/in² at room temperature. DNAase, RNAase, and PMSF (phenylmethylsulfonyl fluoride) (Sigma) were added to the cell lysate to a final concentration of 10 μ g/ml, 5 μ g/ml, and 1 mM, respectively, and the lysate was incubated for 15 min at room temperature. The cell lysate was next centrifuged for 10 min at 16,270 $\times g$ (10,000 rpm; Du Pont SS34 rotor) to remove cell debris and unbroken cells, and the resulting cleared lysate was centrifuged for 1 h at 177,700 $\times g$ (50,000 rpm; Beckman 60 Ti rotor) to pellet the membrane fraction. This fraction was washed twice in buffer A, and the final pellet was resuspended in buffer A to a protein concentration of 15 mg/ml. The membranes were further purified by sucrose gradient centrifugation (12). The purified membranes (1.35 mg of protein per ml) were solubilized by the addition of *n*-octylglucoside (Sigma) (final concentration, 1%). After incubation of the mixture at 4°C for 30 min with gentle stirring, the mixture was centrifuged at 177,700 $\times g$ (50,000 rpm; Beckman 60 Ti rotor) for 1 h at 4°C to remove insoluble material. The supernatant fraction was next concentrated to a protein concentration of approximately 3.4 mg/ml by using a Centriprep-10 apparatus (Amicon).

Enzyme purification. (i) **Sephacose CL 6B-200 (Pharmacia) chromatography.** Fourteen milligrams (3.4 mg/ml) of solubilized membrane protein was applied to a column (30 by 3.0 cm) equilibrated with buffer A. Fractions of 1 ml were collected at a flow rate of 20 ml/h. It was found that ATPase activity eluted in a single peak, and those fractions containing activity were pooled and concentrated by using a Centriprep-10 apparatus.

(ii) **DEAE-Sephacose (Pharmacia) chromatography.** Next,

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7.6 mg (4.8 mg/ml) of protein pooled from the previous step was applied to a column (15 by 2 cm) equilibrated with buffer A, and the column was washed with buffer A until the eluate was free of protein. The bound protein was eluted with a 200-ml 0 to 500 mM linear NaCl gradient in buffer A at a flow rate of 24 ml/h. Fractions (0.6 ml) were collected and assayed for protein and ATPase activity. It was found that the ATPase activity eluted in a single peak at an NaCl concentration of approximately 150 mM. Those fractions containing ATPase activity were pooled.

(iii) **FPLC Mono Q HR 5/5 anion-exchange (Pharmacia) chromatography.** The pooled sample from the previous step was diluted 10-fold in buffer B (buffer A free of glycerol) and then concentrated by using a Centricon PM-10 apparatus (Amicon). The column (1 ml) was equilibrated with buffer B. Following application of 1.0 mg (6.7 mg/ml) of protein, the column was washed with 5 ml of buffer B for 5 min and then with a 35-ml 0 to 1 M linear NaCl gradient in buffer B for 35 min at a flow rate of 1 ml/min. ATPase activity eluted in a single symmetrical peak which coincided with a symmetrical peak of protein. The pooled sample was next concentrated in a Centricon PM-10 apparatus. The purified ATPase could be stored in buffer A at 4 or -20°C without significant loss of activity for at least 1 month.

(iv) **FPLC Superose 6 HR10/30 gel filtration (Pharmacia) chromatography.** Fifty microliters (1.5 mg/ml) of purified ATPase was applied to the column (25 ml), and the sample was eluted in buffer B. Fractions were assayed for both protein and enzyme activity. The protein eluted in a single peak which was coincident with a peak of ATPase activity. The size of the ATPase was estimated based on its elution profile compared with that of thyroglobulin (669 kDa; Sigma), apoferritin (443 kDa; Sigma), and β -amylase (200 kDa; Sigma).

Assays. ATPase activity was determined by measurement of the liberation of P_i . Unless otherwise described, the standard assay mixture with a final volume of 500 μl contained 50 mM sodium acetate (pH 5.0), 5 mM MgCl_2 , and 1 mM ATP. The reaction was initiated by the addition of ATP. After 20 min of incubation at 30°C , the reaction was terminated by the addition of 10% trichloroacetic acid, and the amount of released P_i was determined by the method of Chen et al. (8). For assays to determine ion requirements, the enzyme was first diluted in storage buffer lacking the relevant ion. Protein was measured by the method of Bradford (6).

Amino acid sequencing. The protein to be analyzed was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted to a PVDF (polyvinylidene difluoride) membrane by using a Bio-Rad electroblotting apparatus. After transfer, the protein bands of interest were excised from the membrane and applied to a pulsed liquid-phase protein sequencer with on-line phenylthiohydantoin analyzers (Applied Biosystems) for amino acid sequencing. Analysis was performed in the Genetic Engineering Facility, University of Illinois Biotechnology Center.

RESULTS AND DISCUSSION

Purification of the ATPase. Treatment of purified membranes with *n*-octylglucoside led to efficient release of ATPase activity and a 3.1-fold increase in specific activity. 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and Triton X-100 were also effective in solubilization of the enzyme (89 and 98% yield, respectively). After solubilization, the specific activity of the ATPase increased from 67.3 to 211 nmol

FIG. 1. SDS-PAGE. Lanes 1 to 8, SDS-PAGE with 12% polyacrylamide. Lanes 1 and 8, Sigma SDS-6H molecular mass markers (myosin [205 kDa], β -galactosidase [116 kDa], phosphorylase *b* [97.4 kDa], bovine albumin [66 kDa], egg albumin [45 kDa], carbonic anhydrase [29 kDa]); lane 2, crude membranes; lane 3, purified membranes; lane 4, solubilized proteins; lane 5, fractions from Sepharose CL 6B-200 column; lane 6, fractions from DEAE-Sepharose column; lane 7, purified ATPase from FPLC on Mono Q column. Twenty micrograms of proteins was applied onto each lane. Lane 9, 10% polyacrylamide gel electrophoresis without SDS. Twenty micrograms of the purified *M. voltae* ATPase was applied. SDS-PAGE was carried out as described by Laemmli (26), and protein was visualized by Coomassie brilliant blue R-250 staining.

of P_i per mg of protein per min (activity in membrane and solubilized fractions, respectively).

Further purification of the enzyme by Sepharose CL 6B-200, DEAE-Sepharose, and FPLC Mono Q HR 5/5 anion-exchange chromatography resulted in a preparation whose specific activity was enhanced 108-fold compared with the specific activity of crude membrane. The specific activity of the purified enzyme protein was 4,680 nmol of P_i per mg per min.

To monitor the efficiency of the purification, we subjected material from each stage of the procedure to SDS-PAGE analysis (Fig. 1). The final preparation contained three components whose estimated molecular masses were 80, 55, and 25 kDa. While the enzyme preparation examined in lane 6 has a specific activity that is approximately fourfold higher than the material examined in lane 5, the protein profiles are very similar. This may reflect activation of the enzyme as a result of the DEAE-Sepharose step in the purification. Although we believe that these three polypeptides are subunits of the enzyme complex, this conclusion needs to be confirmed by further biochemical and enzymatic studies. The fact that we observed a single stained band on native gels lacking SDS further demonstrates the purity of the preparation and is a result consistent with the subunit structure. For the sake of identification, we designate the three components α , β , and γ in order of decreasing molecular mass.

In order to estimate the size of the ATPase, the purified enzyme was subjected to Superose 6 HR10/30 gel permeation chromatography on an FPLC system. On the basis of its relative migration compared with that of protein standards, we estimate that the active enzyme has a molecular mass of approximately 505 to 515 kDa. Because the results of a densitometer analysis of the Coomassie brilliant blue-

FIG. 2. Effect of pH on activity of the purified ATPase. One hundred percent activity represents 4,210 nmol of P_i per mg of protein per min. The buffers (50 mM) were sodium acetate (pH 4 to 6) (■), bis-Tris (pH 6 to 7) (○), Tris-Cl (pH 7.5 to 9) (●), and glycine (pH 8 to 10) (▲).

stained bands observed after SDS-PAGE indicated that the three components are present in a ratio of 1:1:1, we consider it possible that the subunits are assembled in the isolated enzyme complex in a molar ratio of $\alpha_3\beta_3\gamma_3$. However, since the method used to estimate the size of the complex can be quite imprecise, our conclusion is tentative. Furthermore, it is possible that components were lost during the isolation procedure or missing because of incomplete solubilization.

Characterization of the ATPase. The activity of both the membrane-associated ATPase and the purified ATPase was maximum near pH 5.0. The data in Fig. 2 are from a representative experiment carried out with the purified enzyme and are similar to those obtained when membrane-associated activity was assayed (data not shown). In assays carried out with both preparations at pH 4.5 and 5.5 activity was typically 50 to 60% and 40 to 60%, respectively, of that observed at pH 5.0 (data not shown). Enzymatic activity required the addition of Mg^{2+} , and the optimal concentration was 5 mM. Neither Ca^{2+} nor Mn^{2+} could substitute for Mg^{2+} . Addition of 20 mM HSO_3^{3-} , HCO_3^{3-} , or SO_4^{2-} had no effect on ATPase activity. Na^+ or K^+ stimulated the ATPase activity slightly, and maximum stimulation (approximately 30%) for either cation was observed when either was present at 40 mM (added as the chloride). The effects of these ions were similar for membrane-associated and purified ATPase. When assayed at a substrate concentration of 1 mM, the purified ATPase hydrolyzed ATP, GTP, ITP, TTP, UTP, and CTP at relative rates of 100, 83, 94, 4.7, 4.4, and 2.2%, respectively. It did not hydrolyze ADP or PP_i .

Several specific inhibitors of other ATPases were examined for their effects (Table 1). Dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol (DES), known inhibitors of membrane-associated F-type and V-type ATPase families (28, 33) through disruption of F_0 and V_0 domain function, inhibited the membrane-associated ATPase activity, while the purified enzyme was entirely refractory to their action. While we cannot rule out the possibility that the observed

TABLE 1. Effect of inhibitors on the *M. voltae* ATPase^a

Inhibitor and concn ^b	ATPase sp act (nmol of P_i /mg of protein/min)	
	Membranes	Purified ATPase
None (control)	63.4	4,310
DCCD		
10	28.1	4,288
30	22.6	4,301
50	17.2	4,276
80	7.9	4,297
100	0.0	4,293
None (control)	61.3	4,100
DES		
10	46.7	4,059
30	34.8	4,096
50	28.0	4,051
80	19.0	4,059
100	15.7	4,060
None (control)	47.3	4,120
KNO_3		
0.06	41.5	2,937
0.10	37.8	2,233
0.60	33.3	1,883
1.00	30.6	1,471
6.00	16.6	906
10.00	9.5	463
20.00	6.4	168

^a Membranes (40 to 70 μ g/ml) or the purified ATPase (5 to 10 μ g/ml) was incubated with the indicated inhibitors at 30°C for 15 min in 50 mM sodium acetate-5 mM $MgCl_2$ buffer (pH 5.0).

^b Concentrations are micromolar for DCCD and DES and millimolar for KNO_3 .

differences in inhibitor sensitivity may be due to the assay of different enzymes, our results are consistent with a structural motif in which the *M. voltae* enzyme has an integral membrane domain which is inactivated by DCCD and DES.

Nitrate, which is generally considered to have a greater specificity in inhibiting V-type ATPases than F-type ATPases (33) was a strong inhibitor of both membrane-associated and purified ATPase. Neither the membrane-associated nor purified ATPase was sensitive to vanadate when present at 10, 50, or 100 μ M whether the assay was performed at pH 5.0, 6.0, 6.5, or 7.0. Vanadate is an inhibitor of the P-type ATPases, which are characterized by the formation of a phosphorylated intermediate (33). Sodium azide (1 mM), an inhibitor of F-type ATPases (28), did not inhibit membrane-associated or pure enzyme. Oligomycin (80 μ g/ml) and ouabain (100 μ M) were without effect.

The macrolide antibiotic bafilomycin A1 has been shown to be a unique inhibitor of V-type ATPases when used in the nanomolar concentration range (4). We observed a 50% inhibition of *M. voltae* ATPase activity by 10 μ M bafilomycin A1 (1 μ mol/mg of ATPase). Approximately 100 μ mol of bafilomycin per mg of ATPase was required for a 90% inhibition of the enzyme (Fig. 3). The concentration range at which bafilomycin A1 inhibited the *M. voltae* enzyme is higher than that reported for those V-type ATPases that have been tested (4). The sensitivity of the *M. voltae* enzyme contrasts to the resistance of F-type ATPases to bafilomycin A1 (4). However, it is possible that the sensitivity of the *M. voltae* enzyme is affected by the pH of our assay. Previous studies examining bafilomycin sensitivity were performed at a pH range of 6.5 to 8.3 (4). We do note that bafilomycin A1 has been shown to inhibit at least some P-type ATPases in

FIG. 3. Effect of bafilomycin A1 on ATPase activity. For membrane preparation, 100% activity represents 67.4 nmol of P_i per mg of protein per min. For the purified ATPase, 100% activity represents 3,920 nmol of P_i per mg of protein per min. Membranes (40 to 70 μ g/ml) (●) or the purified ATPase (5 to 10 μ g/ml) (▲) was incubated with the indicated inhibitor at 30°C for 15 min in 50 mM sodium acetate–5 mM $MgCl_2$ buffer (pH 5.0).

the concentration range in which we have shown it to inhibit the *M. voltae* enzyme (4).

From the pattern of sensitivity that we observed with the ensemble of inhibitors tested, we conclude that the *M. voltae* ATPase has properties most similar to those of ATPases of the V-type family.

We have previously reported the characterization and isolation of another *M. voltae* membrane-associated ATPase (11, 12). On the basis of its sensitivity to vanadate and the identification of a phosphorylated intermediate, we concluded that the enzyme was functionally related to ATPases of the P-type family. We have suggested that this enzyme might be involved in Na^+ extrusion from this marine methanogen. In contrast to the enzyme described in this paper, the *M. voltae* P-type enzyme is resistant to nitrate and bafilomycin and has a pH optimum of approximately 7.5. Furthermore, the membrane-associated form of the P-type enzyme is resistant to DCCD. These results indicate that the membrane-associated activity that we describe here (i.e., assayed at pH 5 and sensitive to DCCD) does not reflect significant activity of the P-type enzyme.

N-terminal amino acid sequence of the α subunit of the *M. voltae* ATPase. Utilizing Edman degradation, we were able to determine 20 amino acid residues from the N terminus of the α subunit (Table 2). We were unable to carry out a similar analysis for the β subunit since its N terminus was apparently modified. When the sequence was compared with the entire protein data base available through the NCBI NIH BLAST E-Mail Server (1), significant sequence similarity to both F-type and V-type ATPase families was observed. In all cases the *M. voltae* sequence matches best with the N-terminal regions of the other ATPases. While the analysis was based on a limited amount of sequence information, the data suggest that for at least the N-terminal region examined, the *M. voltae* enzyme is more similar in sequence to the vacuolar ATPases of the Eukaryota than to the F-type ATPases of either the Bacteria or the Eukaryota. Even the two most similar bacterial ATPases (*Enterococcus* and *Thermus* ATPases) have been classified as members of the V-type family (22, 37).

TABLE 2. Comparison of the N-terminal amino acid sequence of the α subunit of the *M. voltae* ATPase with those of F-type ATPases, V-type ATPases, and other archaeal ATPases (indicated by the source designation A)^a

Organism	Family or source (subunit)	Sequence	Reference
<i>Methanococcus voltae</i>	A (α)	VVGNIKIAGPVVVAEGMKG	20 (this paper)
<i>Halobacterium salinarum</i>	A (α)	DTGEIESVSGPVVTATGLDA	28 (19)
<i>Sulfolobus acidocaldarius</i>	A (α)	SEGRVVRVNGPVIADGMRE	22 (10)
<i>Methanosarcina barkeri</i>	A (α)	VKGETYRVSGPVVTAIGLQA	22 (20)
<i>Methanotheroxiphila</i>	A (α)	VEGRVKRVAGPVVQAVGL	19 (21)
<i>Enterococcus hirae</i>	V (α)	QIGNIIRKVSGLVMAENMSE	21 (22)
<i>Neurospora crassa</i>	V (α)	IHGKIYSVSGPVVVAEDMIG	33 (5)
<i>Gossypium hirsutum</i>	V (69kd)	EYGYVRKVSGLVADGMAG	39 (GenBank)
<i>Daucus carota</i>	V (69kd)	HDNLVRKVSGLVADGMGC	39 (38)
<i>Thermus thermophilus</i>	V (α)	IQGVIOKLAGPAVIAKGMIG	21 (37)
<i>Saccharomyces cerevisiae</i>	V (α)	EYGAISVSGPVVIAENMIG	43 (18)
<i>Prochloron didemni</i>	? (β)	NICKITQVIGPVVDAEFPSG	28 (29)
<i>Plasmodium falciparum</i>	V (α)	EPGVVYKVASGLVIAENMSG	29 (24)
<i>Ipomoea batatas</i> Chloroplast	F (β)	NLGRIVKLIIGPVLDVAFPPG	38 (25)
<i>Synechocystis</i> sp.	F (β)	NVGKITQVIGPVVDAEFPSG	28 (27)
<i>Manduca sexta</i>	V (α)	RFGYVFAVSGPVVTAERMSG	37 (17)
<i>Cytophaga lytica</i>	? (β)	VTGRVSQIIGPVVIDVEFQAG	23 (2)
<i>Vibrio alginolyticus</i>	F (β)	ATGKIVQIIGAVVDVEFP	19 (14)

^a The number immediately to the right of each sequence identifies the residue number of the rightmost amino acid. The GenBank accession number for the *G. hirsutum* sequence is L03186. For *P. didemni* and *C. lytica* the ATPase family has not been reported. Those residues that are identical to those found in the *M. voltae* sequence are shaded.

The BLAST analysis also showed that the *M. voltae* α subunit is most similar in sequence to the α subunits of V-type ATPases. In contrast, it is the β subunit of the F-type ATPases to which the *M. voltae* enzyme showed the highest degree of similarity. The relationship is consistent with previous biochemical studies that indicate that, the A (α) subunits of vacuolar and archaeal ATPases are functionally equivalent to the β subunits of F-type ATPases (9, 10, 19, 20, 34).

Our structural comparison, although limited, is consistent with results obtained with other archaeal membrane-associated ATPases. Thus, amino acid sequence comparison of eukaryotic and eubacterial ATPases to those of the methanogens *Methanosarcina barkeri* and *Methanotherix thermophila*, the sulfur-dependent *S. acidocaldarius*, and the halophile *Halobacterium halobium* have led several investigators to group the ATPases of the Archaea in the V-type family (17, 20–22, 33). This relationship has prompted conjecture concerning possible schemes for the evolution of vacuolar ATPases (16, 32).

We have previously shown that antibody raised against the β -subunit of the ATP synthase of the archaean *S. acidocaldarius* (30, 31) reacts with two *M. voltae* membrane components similar in size to the α and β subunits (see Fig. 8 of reference 15). That result taken together with the results presented in this paper lead us to suggest that the enzyme which we have isolated is the ATP synthase. Nevertheless, it is clear that further studies will be required to determine if this enzyme, indeed, functions in ATP synthesis.

Studies involving whole cells have led to the suggestion that ATP production in *M. voltae* is based on an energetic system in which sodium ions, not protons, are the coupling ions (15). We further proposed that ATP production was mediated by an Na^+ -translocating ATP synthase (15). The isolation of the presumptive ATP synthase now provides the opportunity to investigate the mechanism of energy transduction in this methanogen in more detail. In particular, the ability of this enzyme to participate in proton or sodium ion translocation needs to be explored in vesicular and reconstituted membrane systems.

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