JOURNAL OF BACTERIOLOGY, Sept. 1986, p. 837–841
0021-9193/86/090837-05$02.00/0

Vol. 167, No. 3

Characterization and Purification of the Membrane-Bound ATPase of the Archaebacterium *Methanosarcina barkeri*

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Received 20 February 1986/Accepted 28 May 1986

Membrane-bound ATPase was found in membranes of the archaebacterium *Methanosarcina barkeri*. The ATPase activity required divalent cations, Mg^{2+} or Mn^{2+}, and maximum activity was obtained at pH 5.2. The activity was specifically stimulated by HSO_3^- with a shift of optimal pH to 5.8, and *N,N'*-dicyclohexylcarbodiimide inhibited ATP hydrolysis. The enzyme could be solubilized from membranes by incubation in 1 mM Tris-maleate buffer (pH 6.9) containing 0.5 mM EDTA. The solubilized ATPase was purified by DEAE-Sepharose and Sephacryl S-300 chromatography. The molecular weight of the purified enzyme was estimated to be 420,000 by gel filtration through Sephacryl S-300. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed two classes of subunit, M_r 62,000 (α) and 49,000 (β) associated in the molar ratio 1:1. These results suggest that the ATPase of *M. barkeri* is similar to the F_0F_1 type ATPase found in many eubacteria.

Methanogenic bacteria are strictly anaerobic organisms, and most species are able to grow autotrophically with H_2 and CO_2 as substrates for methanogenesis. On the basis of studies of the 16S RNA sequence, they are considered to belong to the archaebacteria (1). Other properties of methanogens are also different from those of the eubacteria; the membranes of methanogens contain ether-linked polyisoprenoid glycerol lipids (30) and their cell walls lack peptidoglycan (14). Apart from methanogens, the archaebacteria include extreme halophiles and thermoacidophiles.

In the chemiosmotic mechanism of Mitchell (20), a proton motive force established across the cell membrane is a driving force for ATP synthesis. An H^+-translocating ATPase (F_0F_1 ATPase) which catalyzes ATP synthesis has been observed in membranes of both eucaryotes and eubacteria (26). Although membrane-bound ATPase activities were found in membranes of thermoacidophilic archaebacteria, *Thermoplasma* (28), and *Sulfolobus* (32), their properties differed from those of typical F_0F_1 ATPase; their ATPases were insensitive to *N,N'*-dicyclohexylcarbodiimide (DCCD), a specific inhibitor of the F_0F_1 ATPase, and were specifically stimulated by sulfate. The effect of sulfate on their ATPases is considered to be closely associated with their sulfur metabolism. In fact the *Thermoplasma* ATPase appears to serve as a sulfate translocase (28).

ATP synthesis in methanogens is considered to occur by the chemiosmotic mechanism, because ATP synthesis driven by an artificially imposed proton gradient was observed in whole cells of *Methanosarcina barkeri* (23) and *Methanobacterium thermoautotrophicum* (5). The acid-induced ATP synthesis observed in *M. barkeri* cells was inhibited by DCCD (2, 23), indicating the presence of the H^+-translocating ATPase (F_0F_1 ATPase) in *M. barkeri*. ATPase activities have been found in the membranes (5) and cell extracts (15) of some methanogens, although isolation of the enzyme has not been reported so far.

This paper reports the characterization and purification of the membrane-bound ATPase of *M. barkeri* MS. This is the first report on the molecular properties of the methanogen F_1ATPase, and it provides a basis for comparing the F_1 ATPase from archaebacteria with those from other eubacteria.

MATERIALS AND METHODS

Preparation of membranes. *M. barkeri* MS (DSM800) was grown in the methanol-containing medium described by Hippe et al. (13). Membranes were prepared by passing them through a French press as previously described (16), except that the phosphate buffer was replaced by a Tris-maleate buffer.

ATPase activity was assayed by the measurement of P_i released from ATP and other nucleotides. In a typical assay the reaction mixture (0.5 ml) contained 50 mM Tris-maleate buffer (pH 7.0), 2 mM $MgCl_2$, 3 mM ATP, and 0.1 mg of protein. The mixture was incubated at 37°C for 10 or 15 min, and the amount of P_i was measured by the method of Fiske and SubbaRow (10). Protein was determined as described by Lowry et al. (18).

Solubilization and purification of the ATPase. The membrane fraction was suspended in 1 mM Tris-maleate buffer (pH 6.9) containing 0.5 mM EDTA. After incubation at 8°C for 1 h with gentle stirring, the suspension was centrifuged at 100,000 × *g* for 90 min. The supernatant (42 ml, 2.4 mg of protein per ml) was applied to a DEAE-Sepharose column (2.6 by 20 cm) equilibrated with 50 mM Tris-maleate (pH 6.9) (column buffer). After the column had been washed with one column volume of the column buffer, a linear gradient from 0 to 0.6 M NaCl in 50 mM Tris-maleate buffer (pH 6.9) was run at 8°C. The fractions containing ATPase activity were pooled (15 ml), and solid ammonium sulfate was added up to reach 65% saturation. The precipitate obtained after centrifugation at 10,000 × *g* for 20 min was dissolved in a minimal volume of 50 mM Tris-maleate buffer (pH 6.9), and the solution was centrifuged to remove any insoluble material. The supernatant was then applied to a Sephacryl S-300 column (1.6 by 80 cm) equilibrated with the column buffer. The flow rate was 10 ml/min at 8°C, and the fractions with ATPase activity were collected. These were then concentrated in an Amicon ultrafiltration apparatus with an XM-50 membrane. During purification, the ATPase was stable at 4

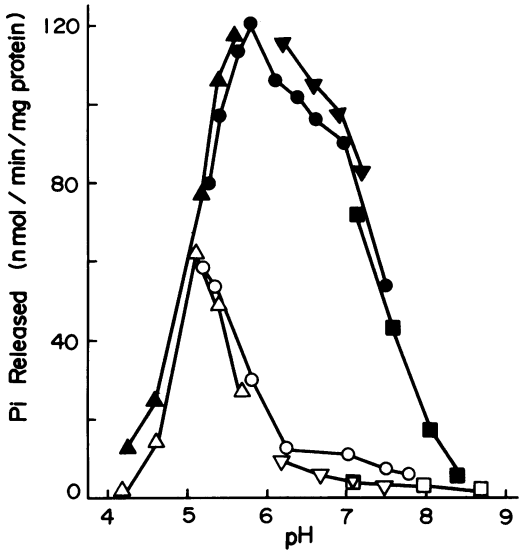

FIG. 1. pH dependence of membrane ATPase of *M. barkeri*. ATPase activity was measured as described in Materials and Methods in the presence (closed symbols) and absence (open symbols) of 20 mM sulfite at the indicated pHs. The pHs were measured before the reaction. Buffers: acetate (▲, △), Tris-maleate (●, ○), PIPES (1,4-piperazinediethanesulfonic acid)-NaOH (▼, ▽), Tris hydrochloride (■, □).

to 8°C and could be stored without loss of activity for at least a week.

The molecular weight of the purified enzyme was determined by gel filtration on a Sephacryl S-300 column (1.6 by 80 cm) with following standards: thyroglobulin, ferritin, catalase, and aldolase.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (17).

Materials. DEAE-Sepharose, Sephacryl S-300, and molecular weight markers for gel filtration were purchased from Pharmacia Fine Chemicals. Molecular weight markers for SDS-PAGE were purchased from Oriental Yeast Co. Oligomycin was purchased from Sigma Chemical Co. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride (NBD-Cl), ouabain, DCCD, and *N*-ethylmaleimide were purchased from Nakarai Chemicals Ltd.

RESULTS

Characterization of membrane-bound ATPase. ATPase activity was found in the 100,000 × *g* pellet of cell extracts of *M. barkeri*. The pellet fraction contains the membranes in which the presence of b-type cytochrome (16) and hydrogenase (24) was reported previously. The activity for ATP hydrolysis was observed at pH 4.7 to 7.0 in the presence of 2 mM Mg^{2+}. The optimum pH was 5.2 (Fig. 1). The enzyme required divalent cations for its activity (Table 1). Mg^{2+} and Mn^{2+} were most effective at both pH 5.2 and pH 7; Co^{2+} and Zn^{2+}, but not Ca^{2+}, could replace Mg^{2+} to some extent. Even in the presence of Mg^{2+}, the monovalent cations K^+ and Na^+ had no stimulatory effect on the enzyme at pH 7, but a slightly inhibitory effect was observed at pH 5.2 (Table 1).

As shown in Fig. 1, the ATPase was active in the low pH range, with an optimum at about pH 5. The optimum pH of 5.2 is below the intracellular pH of about 6.9 (2) and also below the optimum pHs of other cytoplasmic enzymes, for example, pH 6.5 for methyltransferase (31) and sulfite reductase (24). Various attempts were made to activate the *M. barkeri* ATPase at the neutral pH region. Addition of dithiothreitol (1 mM) to the assay mixture, heat treatment at 60°C, and preincubation with trypsin (0.25 mg/ml) at 37°C for 10 min had no effect on enzyme activity. In the case of trypsin treatment, the ATPase activity decreased to 90% of the original level during the incubation.

A variety of anions have been shown to stimulate the mitochondrial ATPase (21). Therefore, the effect of various anions on the *M. barkeri* ATPase was tested (Table 1). Sulfite showed a significant effect on the activity. Figure 1 shows the pH dependence in the presence of 2 mM Mg^{2+} and 20 mM sulfite. The stimulatory effect was observed at pH 5 to 7.5; specific activity at pH 7 increased about 10-fold compared with that in the absence of sulfite. In addition, the optimum pH was shifted from 5.2 to 5.8. The stimulation by sulfite at pH 5 to 7.5 corresponded closely to the concentration of HSO_3^- rather than to the total sulfite or SO_3^{-2} concentration; therefore, the activating species was monoprotonated anion, HSO_3^-. HSO_3^- and HCO_3^- are known to be highly stimulatory to the mitochondrial ATPase. The stimulatory effect of HSO_3^- on the *M. barkeri* ATPase appears to be greater and more specific than that observed in mitochondrial ATPase; HCO_3^- has only a slight stimulatory effect on the *M. barkeri* ATPase. A sulfite-stimulated ATPase was also observed in chromatophores of *Rhodospirillum rubrum* (33).

The membrane-bound ATPase of *M. barkeri* was inhibited by DCCD, a specific inhibitor of the H^+-translocating ATPase (F_0F_1 ATPase) found in bacteria (8) and

TABLE 1. Effect of cations and anions on the membrane ATPase of *M. barkeri*[a]

Addition (mM)	ATPase activity (nmol of P_i/min per mg of protein)	
	pH 5.2	pH 7.0
Divalent cations (2)[b]		
None	<2	0
Mg^{2+}	51.0	11.0
Mn^{2+}	72.9	10.6
Ca^{2+}	0	0
Zn^{2+}	24.3	5.7
Cu^{2+}	10.0	2.0
Co^{2+}	47.4	6.9
Ni^{2+}	15.0	0.4
Monovalent cations[c]		
Na^+ (50)	37.1	11.8
K^+ (50)	40.0	10.9
Na^+ and K^+ (50 and 50)	27.0	10.2
Anions (20)[c]		
HSO_3^-	78.1	81.0
HCO_3^-	—[d]	20.0
SO_4^{2-}	51.5	10.2
SCN^-	48.6	10.2

[a] Membranes were incubated at 37°C for 15 min either in 50 mM Tris-maleate (pH 5.2) or in 50 mM Tris-maleate (pH 7.0) buffer containing 3 mM ATP and the ions indicated above.

[b] Membranes were dialyzed against 50 mM Tris-maleate buffer (pH 6.9) to remove divalent cations.

[c] $MgCl_2$ (2 mM) was added to the mixture described in footnote *a*.

[d] —, Not done.

TABLE 2. Effect of inhibitor on the membrane ATPase of *M. barkeri*[a]

Inhibitor (mM)	ATPase activity (nmol of P_i/min per mg of protein)	% of control
None (control)	81.0	100
DCCD (0.01)	10.3	13
DCCD (0.1)	9.0	11
NaN_3 (1.0)	65.7	81
Oligomycin (0.1)	73.0	90
Ouabain (0.1)	80.8	100
N-Methylmaleimide (0.1)	60.0	74
NBD-Cl (1)[b]	70.7	87

[a] Membranes in 50 mM Tris-maleate (pH 6.9) buffer containing 2 mM $MgCl_2$ were incubated with the indicated inhibitors at 37°C for 10 min, and then the mixture was incubated with 3 mM ATP at 37°C for 15 min. The assay was carried out in the presence of 20 mM sulfite.

[b] Membranes in the same buffer were incubated with 1 mM NBD-Cl at 25°C for 70 min, and then the mixture was incubated as described in footnote *a*.

mitochondria (29). At a DCCD concentration of 10 μM, the ATPase activity decreased to about 10% of the original level at pH 7.0 (Table 2). The inhibition was observed both in the presence and in the absence of HSO_3^- at pH 5 to 7. However, other compounds such as NaN_3, NBD-Cl, *N*-ethylmaleimide, ouabain, and oligomycin did not inhibit the enzyme; NBD-Cl, which is known to inhibit the bacterial F_1 ATPase (7), had no effect on the activity, suggesting the lack of an NBD-Cl-binding site in the *M. barkeri* ATPase.

In addition to ATP, the enzyme could use GTP and UTP as a substrate. ADP and AMP were not hydrolyzed, but ADP inhibited 46% of ATP (2 mM) hydrolysis when it was added at a concentration of 1 mM. No pyrophosphatase activity was observed in the membrane fractions.

Solubilization of the ATPase. The membrane-bound ATPase of *M. barkeri* could be solubilized from the membranes by incubating them in a buffer of low ionic strength in the presence of EDTA, which has been used to solubilize F_1 ATPases from the membranes of most bacteria (7). To solubilize the ATPase, 1 mM Tris-maleate buffer (pH 6.9) containing 0.5 mM EDTA was used. After incubation for 1 h, 57% of the protein and 101% of the ATPase activity were extracted from the membrane (Table 3). The solubilized enzyme was insensitive to DCCD (0.1 mM) at pH 7.

Purification of the solubilized ATPase. The solubilized ATPase was purified by DEAE-Sepharose and Sephacryl S-300 chromatography as described in Materials and Methods. Only one peak of activity was observed during chromatography. The purified enzyme showed a specific activity of 3.3 U/mg of protein and was obtained in a yield of 19% (Table 3).

The molecular weight of the enzyme was estimated to be 420,000 by Sephacryl S-300 chromatography. This value was

TABLE 3. Purification of *M. barkeri* ATPase[a]

Fraction	Protein (mg)	ATPase activity	
		Total (U)[b]	Specific (U/mg)
Membrane	178	17.5	0.10
EDTA extract	100	17.8	0.18
DEAE pool	4.6	11.3	2.5
S-300 eluate	1.0	3.3	3.3

[a] Assays were carried out in the presence of 20 mM sulfite.

[b] One unit of activity is defined as the amount of enzyme liberating 1 μmol of P_i per min.

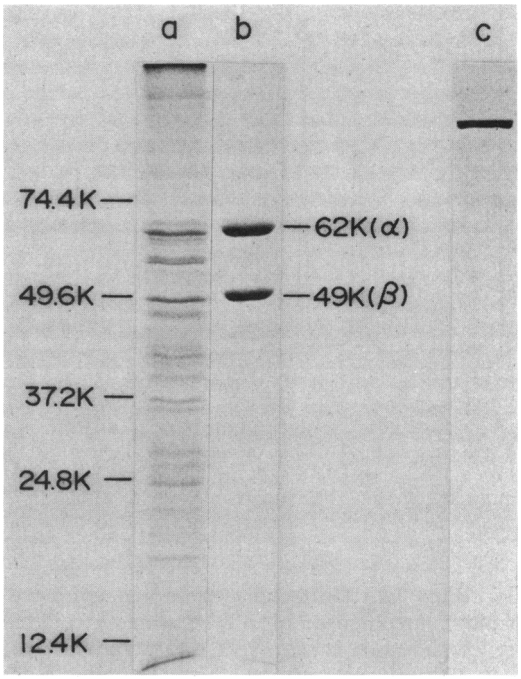

FIG. 2. Protein patterns obtained after PAGE. Lanes: a and b, SDS-PAGE on 12.5% gel; c, 10% gel without SDS. Proteins were stained with Coomassie brilliant blue. Lane a, Membrane fraction (40 μl); lanes b and c, purified ATPase (30 μg). Marker proteins are cytochrome *c*: monomer, 12.4K; dimer, 24.8K; trimer, 37.2K; tetramer, 49.6K; hexamer, 74.4K.

slightly higher than the values determined for other bacterial F_1 ATPases, e.g., 350,000 (350K) to 390K for *E. coli* (9, 11, 26) and 380K for the thermophilic bacterium PS3 (34).

After chromatography on Sephacryl S-300, the purified enzyme was homogeneous on PAGE without SDS (Fig. 2b). SDS-PAGE (12.5% polyacrylamide) of the purified ATPase revealed only two protein bands (Fig. 2c). The molecular weight of these two bands was determined to be 62K and 49K by comparing their electrophoretic mobilities with those of reference proteins (Fig. 2b). In spite of overloading the purified enzyme, other bands were hardly detected on the SDS gel (data not shown). The molar ratio of the two bands was 1:1, estimated from their relative intensities on the SDS gel stained with Coomassie brilliant blue (Fig. 3). Assuming the purified ATPase to be composed of two classes of subunits with molecular weights of 62K (α) and 49K (β), it is estimated that there are four subunits in each molecular weight class, with a total of eight subunits per molecule. This suggests the stoichiometry of the subunits, $\alpha_4\beta_4$, for the solubilized ATPase of *M. barkeri*.

When the purified ATPase was incubated in 50 mM Tris-maleate buffer (pH 7.0) containing Mg^{2+} with membranes depleted of the ATPase, more than 50% of the activity was recovered in the membrane pellet after ultracentrifugation (data not shown), suggesting that the ATPase purified by this procedure has some ability to rebind to the membranes. DCCD sensitivity was also recovered after binding the purified ATPase to the depleted membranes (>50% inhibition at 0.1 mM DCCD).

DISCUSSION

ATP synthesis of methanogenic bacteria has been considered to occur by the chemiosmotic principle of Mitchell (20).

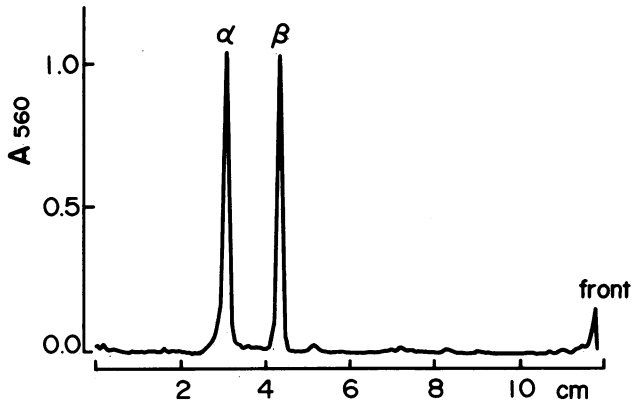

FIG. 3. Densitometric analysis of the purified ATPase on an SDS gel. The gel was stained with Coomassie brilliant blue and then scanned on a Beckman DU-8 spectrophotometer equipped with a gel-scanning attachment. The molar ratio of the subunits α and β was estimated to be 1:1 from the areas of the stained bands.

Recently, Blaut and Gottschalk presented evidence for a chemiosmotic mechanism of ATP synthesis coupled with methane formation in *M. barkeri* (3). The membrane-bound ATPase of *M. barkeri* described here was similar to common F_0F_1 ATPases in the following properties: (i) divalent cation requirement, (ii) DCCD inhibition in the membrane-bound form, (iii) solubilization of the ATPase from the membranes by incubation in a low-ionic-strength buffer with EDTA, and (iv) rebinding of the solubilized ATPase to depleted membrane in a high-ionic-strength buffer with Mg^{2+}. It is apparent that the acid-induced ATP synthesis observed in whole cells of *M. barkeri* (23) is catalyzed by this enzyme.

A membrane-bound, DCCD-sensitive ATPase activity was found in the membrane fractions of thermophilic *Methanobacterium thermoautotrophicum* by Doddema et al. (5) Solubilization of the ATPase was attempted under a variety of conditions, but was not successful. These results suggest that the ATPase activity is not present in cytoplasmic membranes but is present in internal membrane vesicles, which have been observed in *Methanobacterium* species (6, 35), and that the enzyme is localized inside the membrane of these vesicles. Electron-microscopic observations of the cells of *M. thermoautotrophicum* by use of a cytochemical-staining technique also supported this location of the enzyme (6). The *M. thermoautotrophicum* ATPase presumably could not be released from these vesicles because they were closed and present in a right-side-out orientation despite preparation by French pressure cell treatment. *Methanosarcina* species can be distinguished from other methanogens by differences in their metabolism and morphological features (35). As judged from the absence of these vesicles in *Methanosarcina* species, the ATPase of *M. barkeri* is probably localized on the cytoplasmic membranes and thus can be solubilized.

Although ATPase activities were found in the membranes of the thermoacidophilic archaebacteria *Sulfolobus* (32) and *Thermoplasma* (28), their properties differed from those of other typical bacterial F_0F_1 ATPase with respect to their inhibitor sensitivity and substrate specificity. Recently, Mukohata et al. reported the presence of H^+-translocating ATP synthase in *Halobacterium halobium*, but they suggested the existence of a different type of ATP synthase instead of an F_0F_1 ATPase (25). Among archaebacteria, the F_0F_1 type ATPase has been found only in methanogenic bacteria, suggesting that methanogens have evolutionary origins different from those of other groups of archaebacteria with regard to the membrane ATPase.

The solubilized ATPase of *M. barkeri* has the simpler subunit pattern composed of α (62K) and β (49K). In procaryotic and eucaryotic organisms an ATPase complex (F_0F_1) has a similar structure; in general, bacterial F_1 ATPase consists of five different subunits with a stoichiometry of $3\alpha:3\beta:\gamma:\delta:\varepsilon$ (9). On the other hand, ATPases with a more simple subunit structure have also been reported; the F_1 from *Bacillus megaterium* (19) and *Bacillus subtilis* (22) had only two subunits, and *Clostridium pasteurianum* (4) had four subunits as an F_0F_1 complex. The evolution of the membrane ATPase was postulated by Harris on the basis of its subunit structure, inhibitor sensitivities, and amino acid composition (12). According to Harris, the membrane ATPase was initially simple and served as a proton-translocating ATP phosphohydrolase, but in the course of evolution became more complex and developed to function as an ATP synthase. The membrane ATPase of clostridia has been regarded as an early model for the less coplex; it serves in vivo solely as an ATP-driven proton pump (4, 12, 27). *M. barkeri* is metabolically unique among methanogens, since it can use methanol, acetate, and methylamines in addition to H_2 plus CO_2 as methanogenic substrates. Therefore, the results obtained here may not be representative of ATPases from all methanogens in their molecular properties. However, the *M. barkeri* ATPase apparently functions as an ATP synthase that can be driven by an electrochemical proton gradient despite the simple constitution of its F_1 portion.

Methanogenic bacteria are primitive organisms that can reduce CO_2 by H_2 and produce methane as an endproduct of their metabolism. This is consistent with the origin of primitive organisms which appeared under the early anaerobic atmosphere of the earth. If methanogens are phylogenetically old organisms, the membrane ATPase of *M. barkeri* would suggest another model for the early F_0F_1 complex, which might already function as an ATP synthase in vivo in an early stage of the evolution.

Further isolation of the whole F_0F_1 complex, including its reconstitution to liposomes, is now in progress.

ACKNOWLEDGMENTS

I am grateful to S. Isoda and S. Shiono for helpful advice.

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