Characterization of a P-type ATPase of the Archaeabacterium

*Methanococcus voltae*

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Rita M. Dharmavaram and Jordan Konisky‡

From the Department of Microbiology, University of Illinois, Urbana, Illinois 61801

The vanadate-sensitive ATPase of *Methanococcus voltae* has been purified by a procedure which includes, purification of the cytoplasmic membrane by sucrose gradient centrifugation, solubilization with Triton X-100, and DEAE-Sephadex and Sephacryl S-300 chromatography. While the DEAE-Sephadex step provided a preparation consisting of two polypeptides (74 and 52 kDa), the Sephacryl S-300 step yields a product with a subunit of 74 kDa. Incubation of either membranes or purified ATPase with [$\gamma$-32P]ATP followed by acidic (pH 2.4) lithium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated the vanadate-sensitive labeling of a 74-kDa acyl phosphate intermediate. These results indicate that the *M. voltae* ATPase is of the P-type.

The methanogen *Methanococcus voltae* is a strictly anaerobic archaeabacterium that derives energy from reduction of CO$_2$ by H$_2$ or formate to form methane (1). Since uptake of amino acids in this methanogen has been shown to be coupled to a sodium gradient (2), it is important to establish the underlying mechanism whereby energy generated in reactions leading to methane formation is transduced to form ion gradients. The characterization of ion-motive ATPases in this organism is, therefore, relevant and important.

Ion-motive ATPases are divided into three major categories. The P-type ATPases are sensitive to vanadate and form an acyl phosphate intermediate during ATP hydrolysis (3). We had previously suggested the presence of such an ATPase in *M. voltae* (4) based on sensitivity to vanadate. Such P-type ATPases have been characterized in *Schizosaccharomyces pombe* (5), *Neurospora crassa* (6), *Escherichia coli* (7), *Streptococcus faecalis* (8), and animal cells (9, 10). These ATPases are usually composed of monomeric or multimeric complexes of a single catalytic polypeptide of about 100 kDa. They function physiologically in the direction of ATP hydrolysis and vary with respect to cation specificity.

The second category, F-type ATPases, are inhibited by N,N'-'dicyclohexylcarbodiimide (DCCD) (11) and azide (11). These ATPases function physiologically as ATP synthases in mitochondria (12), chloroplasts (13), and respiring bacteria (14). They have a complex structure consisting of a multisinus-
unbound protein with 15 ml of buffer. The bound protein was eluted by a 200-ml, 0-500 mM KCl gradient at a flow rate of 2.5 ml/h. Fractions of 2.5 ml were collected and assayed for protein and for ATPase as described below.

**Sephacryl S-300 Chromatography**—1% octyl glucoside was added to 5.32 mg of the Triton X-100-solubilized membrane protein to disperse micelles. This was followed by dialysis against two changes of TMG buffer lacking 1% Triton X-100. Fractions of 0.2 ml were collected at a flow rate of 2 ml/h. The same column was run three more times using 0.2 ml of the concentrate each time, and the fractions from each run were collected on top of those from the previous run. The ATPase activity was assayed as described below. The S-300 column was calibrated by the following marker proteins: ferritin (450 kDa), catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and cytochrome c (12.4 kDa).

**Phosphorylation of M. voltae Membranes**—Phosphorylation was performed by the method of Post and Sen (30). The labeling was carried out at 0 °C in a 1-ml reaction volume containing 40 μM [γ-32P]ATP (450 Ci/mmol), 40 μM MgCl2, 30 mM Tris-Cl (pH 7.5), 2 mg of carrier bovine serum albumin, and 100-120 μg of native membranes. The reaction was initiated by the addition of radioactive ATP with constant stirring and terminated at the appropriate time by the addition of 25 ml of ice-cold 10% trichloroacetic acid containing 40 mM NaH2PO4, 5 mM Na2HPO4, and 1 mM ATP. The quenched reaction mixture was centrifuged at 27,000 × g for 15 min at 0 °C in a Sorvall SS-34 rotor. The supernatant was poured off and the inside of the tube was wiped with a tissue to remove the remaining supernatant. The pellet was suspended in 1 ml of 30 mM HCl with chilled glass rods and incubated at room temperature for 30 min. At that time 9 ml of 30 mM HCl was added and the pellet was recovered by the centrifugation step described above.

**Lithium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—This was performed as described by Fairbanks and Avruch (31) except that the lithium salt of dodecyl sulfate (LDS) was used (32). Slab gels (5.6%) buffered to pH 2.4 with 50 mM Tris citrate contained 0.2% LDS. The gels were polymerized on Gel Bond PAG film to facilitate handling. The membrane pellet obtained from the last centrifugation step described above was disaggregated in 1% LDS, 50 mM Tris buffer (pH 2.4), 2% β-mercaptoethanol, 4 M urea, 20% glycerol, and 10 μg/ml pyronin Y and incubated at room temperature for 15 min. The gels were electrophoresed at 150 V for 3-4 h at 4 °C and immediately dried on blotter paper and autoradiographed with Cronex Lightning Plus intensifying screen for 24-72 h at −70 °C.

**Fluorescein 5'-isothiocyanate Labeling**—M. voltae membranes were incubated with varying concentrations of FITC (dissolved in dimethyl sulfoxide) at 37 °C in 30 mM Tris borate (pH 9.2) and 1 mM EDTA for 40 min. The reaction was stopped by a 10-fold dilution into the ATP assay buffer containing 20 mM Tris-Cl (pH 7.5), 5 mM MgCl2, and 10% glycerol. An identical set of reactions to which 5 mM ADP or 5 mM ATP was added before the addition of FITC was run simultaneously.

**Assays**—The ATPase was assayed by the release of inorganic phosphate as described previously (4). Solutions of bafilomycin A1 were prepared in dimethyl sulfoxide as described previously (15). 20 μg of protein was used in the bafilomycin A1 experiment. Protein was measured by the modified Lowry assay (33).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—This was performed as described by Laemmli (34) and the gels were stained with Coomassie Brilliant Blue R-250.

**Materials**—Sodium orthovanadate was purchased from Sigma. [γ-32P]ATP was from ICN Radiochemicals. DEAE-Sephadex and Sephacryl S-300 were from Pharmacia LKB Biotechnology Inc. Bafilomycin A1 was obtained from Dr. K. Altendorf (Universitat Osnabrück) and beet tonoplast ATPase from Dr. D. P. Briskin (University of Illinois).

**RESULTS AND DISCUSSION**

**Purification of the ATPase**—The specific activity of the sucrose gradient purified membranes was determined to be 0.25 pmol of P$_i$/min/mg of protein. The addition of 1% Triton X-100 solubilized 100% of the enzyme activity present in the membranes and resulted in a specific activity of 0.44 pmol of P$_i$/min/mg of protein. This corresponds to a 106% recovery of ATPase and a 1.8-fold purification.

When anion exchange chromatography with DEAE-Sephadex was performed, a single peak of vanadate-sensitive ATPase eluted at around 200 mM KCl (Fig. 1). The pooled ATPase containing fractions from this step had a specific activity of 3.00 μmol of P$_i$/min/mg of protein. The result was an additional 6.8-fold purification of the enzyme and an overall recovery of 25%. The SDS-polyacrylamide gel electrophoresis analysis of ATPase activity containing fractions revealed that DEAE-Sephadex chromatography was an efficient purification step as these fractions contained essentially only two polypeptides (74 and 52 kDa, Fig. 2).

Sephacryl S-300 gel filtration was also utilized to purify the active ATPase. Membranes subjected to sucrose gradient centrifugation had a specific activity of 0.5 μmol of P$_i$/min/mg of protein. Extraction with 1% Triton X-100 resulted in a specific activity of 1.05 μmol of P$_i$/min/mg of protein corresponding to a 124% recovery of the ATPase and a 2.1-fold purification. The extraction process was followed by addition of 1% octyl glucoside to disperse micelles, dialysis to remove detergents, and Sephacryl S-300 gel chromatography. The pooled ATPase containing fractions from the final step had a specific activity of 17.5 μmol of P$_i$/min/mg of protein. This resulted in an additional 16.6-fold purification of the ATPase and an overall recovery of 73.8%. It was observed that the ATPase eluted close to ferritin suggesting a molecular weight of 205 kDa.
mass of about 450 kDa (data not shown). However, we cannot rule out the possibility of incomplete removal of detergent, and definitive assignment of the molecular mass of the holoenzyme must await further studies. Unlike the pooled fractions from DEAE-Sephadex chromatography, SDS-polyacrylamide gel electrophoresis analysis of the purified ATPase from the Sephacryl S-300 column demonstrated a single protein of 74 kDa. Although we cannot exclude the possibility that the 52-kDa subunit observed after chromatography on DEAE-Sephadex is an unrelated contaminant, it is possible that it is a regulatory or structural (e.g. involved in anchoring the catalytic subunit to membrane) component of the ATPase.

Phosphoenzyme Formation—When the M. voltae ATPase fractions at the various steps of purification were incubated with [γ-32P]ATP, a 74-kDa protein was phosphorylated (Fig. 3). This protein was not labeled when the pooled ATPase fractions from DEAE-Sephadex were incubated with 50 μM vanadate before the addition of [γ-32P]ATP (Fig. 3, lane 4). The radioactivity at the base of the gel is observed even when the enzyme is boiled or treated with protease before labeling (data not shown). We have also noticed that the amount of radioactivity in the wells and at the base seems to vary with different batches of radioactive ATP.

When the Sephacryl S-300 purified M. voltae ATPase was phosphorylated and then treated with 0.25 M hydroxylamine (pH 5.2) after the trichloroacetic acid precipitation step, label was no longer associated with the 74-kDa protein (Fig. 3, lane 6). This result conclusively rules out the presence of phosphoserine or phosphothreonine intermediates which are both stable at acid pH and resistant to hydrolysis by hydroxylamine (35).

The chemical nature of the phosphoryl ATPase linkage was further investigated by determining the stability of the intermediate at different pH values (Fig. 4). M. voltae membranes were phosphorylated, trichloroacetic acid-precipitated and suspended in buffers of varying pH. It can be seen that the amount of radioactivity remaining associated with the 74-kDa protein decreases as the pH becomes more alkaline. The instability of the intermediate at alkaline pH makes it unlikely that it is a phosphohistidine or phosphohistidyl intermediate (35). The pH stability profile is similar to the N. crassa plasma membrane ATPase which is also unstable at alkaline pH and exists as an acyl phosphate (6). Hence, the M. voltae acyl phosphate may well be an aspartyl derivative.

Another characteristic of the P-type ATPases is the rapid turnover of the phosphorylated intermediate. When M. voltae membranes were labeled for different times, it was determined by densitometry of the resulting autoradiograms that the labeling was essentially complete in 2.5 s (the shortest time tested, data not shown). Evidence for the rapid turnover of the intermediate was provided by a pulse-chase experiment (Fig. 5). M. voltae membranes were phosphorylated for 2 min with [γ-32P]ATP, cold 5 mM ATP was added, and the 20-s chase was terminated by the addition of trichloroacetic acid.
Insensitivity to Bafilomycin A₁ and NO²⁻.—The macrolide antibiotic bafilomycin A₁ inhibits a number of P-type ATPases: for example, the Kdp ATPase of *E. coli*, Na⁺⁺-K⁺⁺-ATPase of ox brain, Ca⁺⁺-ATPase of sarcoplasmic reticulum, and H⁺⁺-ATPase of Neurospora in the micromolar range and the V-type ATPases from *Neurospora* vacuoles, chromaffin granules, and *Zea mays* in the nanomolar range (15).

However, bafilomycin A₁ failed to inhibit the membrane-bound or Triton X-100 solubilized *M. voltae* ATPase up to a concentration of 1 mM (Fig. 6). This contrasted the results seen for beat tonoplast ATPase that was completely inhibited at a concentration of 1 µM. Our results are similar to those observed in the case of the P-type ATPase of *S. faecalis* which is not inhibited by bafilomycin A₁ up to a concentration of 1 mM (15). Clearly sensitivity to bafilomycin A₁ would seem not to be a firm diagnostic tool for the identification of P-type ATPases.

We also observed a lack of inhibition of *M. voltae* ATPase activity by 100 mM KNO₃ an inhibitor of V-type ATPases. This result was in contrast to that of beat vacuolar ATPase which was 85% inhibited by 100 mM KNO₃.

This is the first report of the purification of a vanadate-sensitive ATPase that forms an acyl phosphate intermediate in a member of the archaeobacterial kingdom. Future delineation of structure, biochemistry, and function of *M. voltae* ATPase together with characterization of its structural gene will both clarify its role in methanogen energetics and further our understanding of the molecular mechanisms and evolution of these enzymes.

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REFERENCES

M. voltae P-type ATPase