Soluble P-type ATPase from an archaeon, *Methanococcus jannaschii*

Haruo Ogawa[a], Tatsuya Haga[b], Chikashi Toyoshima[a,*]

[a] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo, Japan*
[b] *Department of Neurochemistry, Faculty of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan*

Received 2 February 2000; received in revised form 14 March 2000

Edited by Matti Saraste

Abstract MJ0968 has been proposed to be an ancestor of P-type ATPase, because its primary structure is highly homologous to that of the core catalytic domain of P-type ATPase. However it completely lacks amino acid sequences that possibly constitute transmembrane domains. To examine if MJ0968 is indeed a P-type ATPase, it was overexpressed in *Escherichia coli* and purified. It did show ATPase activity, autophosphorylation and inhibition by vanadate. All these properties support the idea that MJ0968 is indeed a soluble P-type ATPase.

Key words: P-type ATPase; Cloning; Overexpression; Ion pump; *Methanococcus jannaschii*

1. Introduction

P-type ATPases exist from bacteria to mammalian and play crucial roles in transporting ions and establishing electrochemical ion gradients across biological membranes [1–3]. All members of P-type ATPase so far known have the same overall architecture, with one ATP binding domain and 6–10 transmembrane α-helices forming ion pathways [4]. In spite of the physiological importance of P-type ATPases and a number of biochemical studies, little is known as yet about their structure–function relationships. What kind of conformational changes are involved in ATP hydrolysis? How is the signal of the ATP hydrolysis transmitted to the transmembrane region? To answer such questions, high-resolution structures in different physiological states are needed. Given the well-known difficulty in crystallization of membrane proteins, crystallizing the soluble domain alone will be a viable alternative. This approach has particular advantages if one aims at crystallization of the enzyme in different conformations and of mutants. Though overexpression of the soluble domains of P-type ATPases has already been performed, crystallization has been unsuccessful presumably due to problems in the refolding process of denatured proteins [5,6] or cleavage of fusion proteins [7].

Methanococcus jannaschii is the first archaeon whose complete genomic sequences are determined [8]. Many structural studies have already been performed as a part of 'structural genomics' [9,10]. The MJ0968 gene encodes a sequence of 273 amino acids that is highly homologous to the core catalytic domain of P-type ATPase [11]. However, unlike authentic P-type ATPases, MJ0968 has no putative transmembrane domains and must be soluble (Fig. 1).

In order to characterize MJ0968, we expressed MJ0968 in *Escherichia coli* and examined if it is a P-type ATPase. We report here that MJ0968 is indeed a soluble P-type ATPase and suitable for structural studies.

2. Materials and methods

2.1. Subcloning the MJ0968 gene

The pUC18 plasmids harboring AMJEP60, a genomic fragment containing the MJ0968 gene were obtained from the American Type Culture Collection. The coding region of the MJ0968 gene was amplified by polymerase chain reaction (PCR) using 5′-sense oligonucleotide primer containing a restriction site for *Bam*HI (5′-GCGGATCCATGAAAGTGGCTATA- GTGTTTGAC-3′, the restriction site is underlined) and a 3′-reverse complement primer with *Xho*I site (5′-CCGGATCCCTCGAGTCAAGAACACTCCTTAAT-TTGCATTC-3′). The PCR product was digested with *Bam*HI and *Xho*I, ligated into the vector pGEX-6P-3 (A GST fusion vector with the recognition site for PreScission Protease (PsP), Amersham Pharmacia), cut with the same restriction enzymes and transformed into *E. coli* DH5α for DNA sequencing and BL21 for DNA expression.

2.2. Overexpression of MJ0968 in E. coli

The transformed BL21 was grown in 2×TY medium with 50 μg/ml of ampicillin at 37°C until the optical density at 600 nm reached 0.9. The incubating temperature was then reduced to 20°C and the production of the recombinant protein was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. The lower temperature (20°C) after induction was effective in keeping MJ0968 in the soluble fraction. The cells were harvested 24 h later by centrifugation and stored at −80°C until needed.

2.3. Purification of MJ0968

Frozen cells (from 2 l of culture) were thawed and resuspended in 100 ml of buffer A (20 mM MOPS, pH 7.0, containing 1 mM EDTA, and 60 μg/ml lysozyme) and incubated for 1 h at 4°C. The cells were broken by mild sonication (30 s on and 2 min off for three times, using Tomy ultrasonic disrupter UD-200) and the supernatant was separated from cell debris by ultracentrifugation (150 000×*g* for 1 h). The supernatant was mixed with 10 ml of Glutathione-Sepharose 4B (Amersham Pharmacia) previously equilibrated with buffer B (20 mM MOPS, pH 7.0, containing 100 mM NaCl) for 1 h. Then they were packed into a column (1.5 cm diameter×10 cm length). The column was washed with five volumes of buffer B and further with five volumes of buffer C (50 mM MOPS, pH 7.0, containing 150 mM NaCl, 1 mM EDTA, and 1 mM DTT). Five ml of cleavage buffer (buffer C containing 200 units of PsP) was then added to the column and left to stand for 12 h at 4°C. The eluate with buffer C was collected as 2.5 ml fractions. Fractions were analyzed by SDS–PAGE and those containing MJ0968 were pooled and concentrated by ultrafiltration (Amicon, Centriprep YM10). Protein concentration was determined by the BCA method (Pierce) with BSA as the standard.

2.4. ATPase assay of MJ0968

The ATPase activity of MJ0968 was measured in two different ways. To examine the effect of temperature and orthovanadate, liberated P_i from ATP was measured. Purified MJ0968 (10 μg) was left to react in a 100 μl reaction mixture I (50 mM HEPES, pH 7.4, containing 50 mM NaCl, 4 mM $MgCl_2$, and 2 mM ATP). The reaction

*Corresponding author. Fax: (81)-3-5841 8491.
E-mail: ct@iam.u-tokyo.ac.jp

PII: S0014-5793(00)01374-0

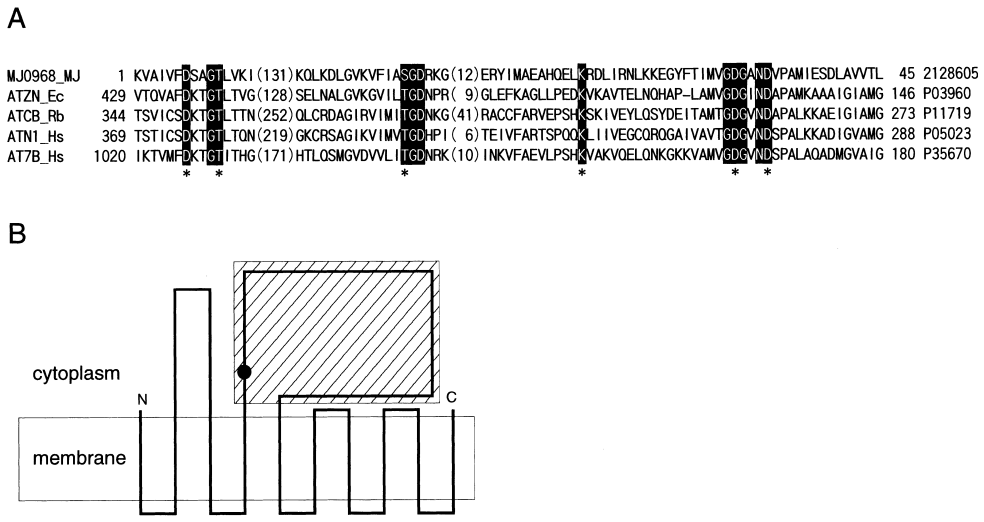

Fig. 1. Architecture of MJ0968. A: Multiple alignment of the P-type ATPases and MJ0968. MJ0968 is highly homologous to the core catalytic domains of P-type ATPases. The abbreviations are: MJ0968_Mj, MJ0968 from *M. jannaschii*; ATZN_Ec, Zn^{2+}-ATPase from *E. coli*; ATCB_Rb, SR Ca^{2+}-ATPase from rabbit; ATN1_Hs, Na^{+},K^{+}-ATPase α_1 subunit from *Homo sapiens*; AT7B_Hs, Cu^{2+}-ATPase 2 (Wilson disease associated protein) from *Homo sapiens*. SWISS-PROT or GenPept accession codes are listed in the rightmost column. Conserved amino acid residues are shaded. The marked residues (*) are thought to be critical for catalytic activity [11]. B: Schematic diagram for the topology of the P-type ATPase. Shaded region represents the core catalytic domain; MJ0968 consists of this region only. The circle indicates the residue of phosphorylation (D8 in MJ0968). The numbers indicate those of inserted amino acid residues.

was started at each temperature (25, 37, 50, 60, 70°C) by the addition of reaction mixture I, and aliquots were taken at every 10 min for 60 min and put into ice for termination of the reaction. Inorganic phosphate in the reaction mixture was measured enzymatically with Iatro–Chrom P_i–S (Iatron Lab.). To evaluate the K_m value, the coupled enzyme method [12] was used with slight modifications. Purified MJ0968 (10 μg) was incubated in 800 μl of reaction mixture II (50 mM HEPES, pH 7.4, containing 50 mM NaCl, 4 mM $MgCl_2$, 0.2 mM NADH, 0.5 mM phospho-enol-pyruvate, 18 units lactic dehydrogenase, 24 units pyruvate kinase, and 0.4 μM to 2 mM ATP) at 37°C. The decrease of optical density at 340 nm was continuously monitored to measure the ATPase activity. In either method, spontaneous hydrolysis of ATP was always measured for compensation.

2.5. Autoradiography of MJ0968

Purified MJ0968 (5 μg) was reacted in 30 μl of a solution containing 50 mM HEPES, pH 7.4, 50 mM NaCl, 4 mM $MgCl_2$, and 0.1 mM [γ-^{32}P]ATP at 25, 37, and 50°C. The reaction was stopped by mixing two volumes of chilled 20% (w/v) trichloroacetic acid solution containing 50 mM HEPES, pH 7.4. The acid-denatured proteins were sedimented by centrifugation for 30 min at 15000×*g* at 4°C. The pH of the pellet was neutralized by the addition of 1 μl of 1 M Tris-base and then suspended in SDS–PAGE sample buffer. The suspended pellets were subjected to SDS–PAGE (12%) for 2 h. After electrophoresis, the gel was fixed in 45% methanol and 10% acetic acid for 10 min and then dried. X-ray film was exposed to the dried gel for 2 days.

3. Results

3.1. Subcloning, expression, and purification of MJ0968

The MJ0968 gene from AMJEP60 was subcloned and inserted into pGEX-6P-3 immediate downstream of the PsP cleavage site. Expression of MJ0968 in *E. coli* BL21 resulted in the appearance of a new band on an SDS gel (Fig. 2). The purification scheme outlined in the previous section yielded ~3 mg of MJ0968 from 2 l of culture. The purified protein formed a single band of 30 kDa M_r on an SDS gel, in agreement with the predicted value of 30.7 kDa from the DNA sequence (Fig. 2).

3.2. Kinetic characterization of MJ0968

MJ0968 has been proposed as a homologue of the P-type ATPase by sequence alignment (Fig. 1) [11]. As a preliminary characterization, its ATPase and GTPase activities were measured at 37°C. With 2 mM of substrate in the presence of 4 mM $MgCl_2$, the ATPase activity was 0.13 μmol P_i/min/mg of protein and the GTPase activity was ~60% of ATPase

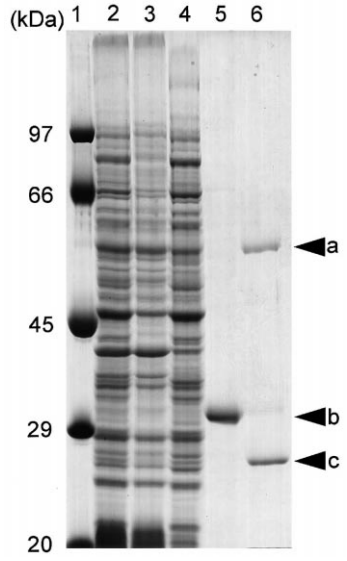

Fig. 2. Purification of MJ0968. SDS–PAGE of fractions obtained in various stages of purification. Coomassie Blue staining. Lane 1: Molecular mass standards (the molecular masses are indicated in kDa); lane 2, crude extract; lane 3 and 4, membrane and cytoplasmic fractions, respectively, derived from the crude extract; lane 5, eluate after PsP digestion; lane 6, eluate with glutathione solution. Arrowheads indicate uncleaved GST-MJ0968 fusion protein (a), MJ0968 (b), and GST (c).

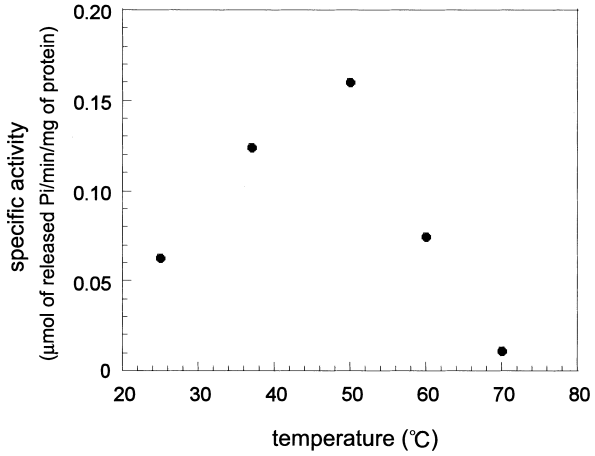

Fig. 3. Effect of temperature on ATPase activity of MJ0968. ATPase activity of purified MJ0968 at various temperatures (25, 37, 50, 60, 70°C). Each point shows the mean value of three experiments.

activity, typical to P-type ATPases. Mg^{2+} was required for the ATPase activity, showing that Mg^{2+}–ATP is the substrate. Ca^{2+} could substitute Mg^{2+} only partially, resulting in lower (~50%) activity under the same conditions. There was no dependence found on Na^+ or K^+.

As the next step, we examined the temperature dependence considering its thermophilic origin (Fig. 3). MJ0968 showed maximum activity (0.16 μmol of released P_i/min/mg of protein) at 50°C, but only little at 70°C.

The Michaelis constant (K_m) and V_{max} at 37°C were calculated from the Eadie–Hofstee plot (Fig. 4). As seen in Fig. 4, the plot could be fitted by two straight lines intersecting at ~120 μM ATP. The apparent K_m values obtained were 11.4 μM (lower ATP concentration) and 81.7 μM (higher ATP concentration). Such dependence on the ATP concentration was also found with other P-type ATPases (for example, K_m of 3.3 μM and 181 μM were reported for Na^+,K^+-ATPase)

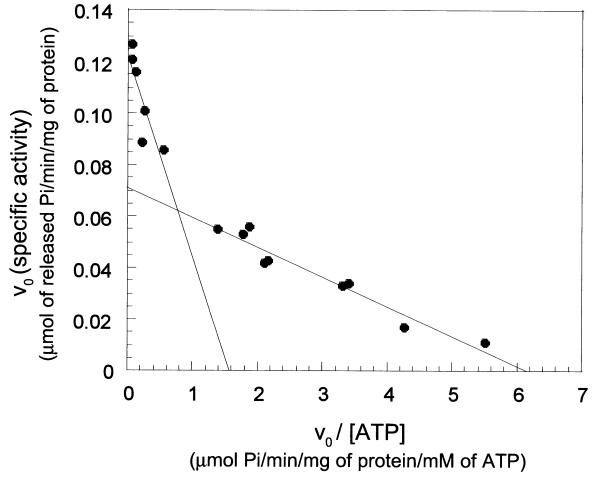

Fig. 4. Eadie–Hofstee plot for ATPase activity of MJ0968. The steady state rate of ATP hydrolysis at 37°C for various ATP concentrations. The ATPase activity was measured using an enzyme coupled assay. The apparent Michaelis constants (K_m) obtained with two approximating lines were 11.4 μM (lower ATP concentration) and 81.7 μM (higher ATP concentration). Each point represents the average of three experiments.

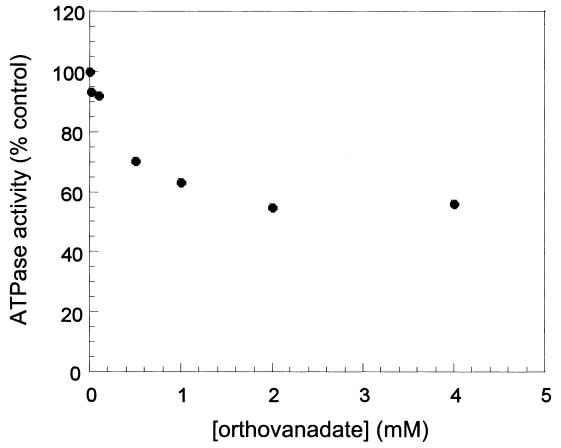

Fig. 5. Inhibition of ATPase activity of MJ0968 by orthovanadate. The ATPase activity of MJ0968 at 37°C in the presence of orthovanadate was determined by measuring liberated P_i for 60 min after 10 min of pre-incubation. Each point represents the average of three experiments.

[13]. The obtained number for V_{max} was 0.13 (μmol P_i/min/mg of protein), which yields a turnover rate of 4 per min.

Orthovanadate is a common inhibitor to P-type ATPases [14], and certainly inhibited the activity of MJ0968 (Fig. 5). Although the enzyme showed 50% activity even at 4 mM, this is again consistent with observations obtained with Ca^{2+}-ATPase (~50% activity at 4 mM orthovanadate [15]).

3.3. Autoradiography of MJ0968

P-type ATPases form phosphorylated intermediates during their reaction cycle [16]. We tried to detect a phosphorylated state of MJ0968 by autoradiography. Clear bands were observed at ~30 kDa molecular weight as expected (Fig. 6). Consistent with the higher activity at higher temperatures, the bands appeared denser at higher temperatures.

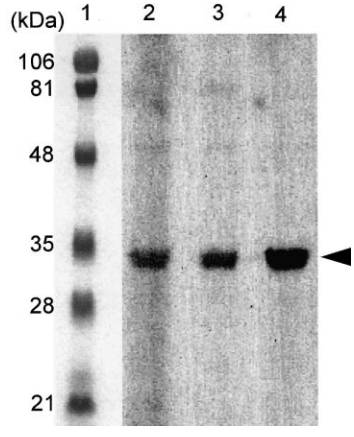

Fig. 6. Autoradiography of MJ0968. Purified MJ0968 (5 μg) incubated with 0.1 mM [γ-^{32}P]ATP at various temperature (25, 37, 50°C) were subjected to autoradiography as described in Section 2. Lane 1: molecular mass standards (numbers in kDa); lane 2, 25°C; lane 3, 37°C; lane 4, 50°C. The arrowhead indicates the expected position of MJ0968.

4. Discussion

In this study we have shown that (i) MJ0968 possesses ATPase activity, (ii) it forms phosphorylated intermediates in its catalytic cycle, (iii) the ATPase activity is partially inhibited by orthovanadate, and (iv) its K_m values (11.4 and 81.7 μM) are appropriate for a P-type ATPase. These findings corroborate the idea that MJ0968 is a unique P-type ATPase that has no transmembrane domains.

Several reports described the overexpression of soluble domains of P-type ATPases in *E. coli* [5–7]. Though they have demonstrated the binding of nucleotides, they have not apparently succeeded in obtaining large amounts of fully active enzymes. On the other hand, MJ0968 is a soluble P-type ATPase by itself; this must be a great advantage for crystallization and mutational analysis. We have already succeeded in producing functional MJ0968 in *E. coli*, and in obtaining large quantities of pure soluble protein (1.5 mg from 1 l culture).

Though *M. jannaschii* lives at high temperature (the optimum temperature is 85°C), MJ0968 showed little ATPase activity at 70°C or above. Also the turnover rate was very low (less than 1/100 of that for Ca^{2+}-ATPases). One possible explanation for this fact is that MJ0968 needs partners for full activity and stability. It certainly lacks the N-terminal soluble domain (so called 'β-strand' domain) that constitutes ~20% of the soluble domain of P-type ATPases (Fig. 1). It is also likely that there exists a separate transmembrane partner. For example, ABC transporters contain four structural domains: two nucleotide-binding and two transmembrane domains [17]. In eukaryotes these four domains are generally fused into a single polypeptide chain, whereas, in prokaryotes, they tend to form separate subunits that are assembled into a membrane-bound complex [17]. Thus it is tempting to hypothesize that MJ0968 is a primeval P-type ATPase: with unknown partners, it may work as an ion pump in *M. jannaschii*.

Acknowledgements: We thank Ms. N. Ueyama for her technical assistance. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture (to C.T.) and from Japan Science and Technology Corporation (CREST) (to T.H.).

References

[1] Skou, J.C. and Esmann, M. (1992) J. Bioenerg. Biomembr. 24, 249–261.
[2] Bull, P.C. and Cox, D.W. (1994) Trends Genet. 10, 246–252.
[3] Skou, J.C. (1988) Methods Enzymol. 156, 1–25.
[4] Møller, J.V., Juul, B. and le Maire, M. (1996) Biochim. Biophys. Acta 1286, 1–51.
[5] Gatto, C., Wang, A.X. and Kaplan, J.H. (1998) J. Biol. Chem. 273, 10578–10585.
[6] Moutin, M.J., Cuillel, M., Rapin, C., Miras, R., Anger, M., Lompre, A.M. and Dupont, Y. (1994) J. Biol. Chem. 269, 11147–11154.
[7] Obsil, T., Merola, F., Lewit-Bentley, A. and Amler, E. (1998) Gen. Physiol. Biophys. 17 (Suppl 1), 52–55.
[8] Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D., Kerlavage, A.R., Dougherty, B.A., Tomb, J.F., Adams, M.D., Reich, C.I., Overbeek, R., Kirkness, E.F., Weinstock, K.G., Merrick, J.M., Glodek, A., Scott, J.L., Geoghagen, N.S.M. and Venter, J.C. (1996) Science 273, 1058–1073.
[9] Kim, S.H. (1998) Nat. Struct. Biol. 5 (Suppl.), 643–645.
[10] Kim, K.K., Kim, R. and Kim, S.H. (1998) Nature 394, 595–599.
[11] Aravind, L., Galperin, M.Y. and Koonin, E.V. (1998) Trends Biochem. Sci. 23, 127–129.
[12] Anderson, K.W. and Murphy, A.J. (1983) J. Biol. Chem. 258, 14276–14278.
[13] Kanazawa, T., Saito, M. and Tonomura, Y. (1970) J. Biochem. (Tokyo) 67, 693–711.
[14] Macara, I.G. (1980) Trends Biochem. Sci. 5, 92–94.
[15] Aureliano, M. and Madeira, V.M. (1994) Biochim. Biophys. Acta 1221, 259–271.
[16] Pederson, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 146–150.
[17] Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E. and Higgins, C.F. (1990) Nature 346, 362–365.