The *Methanocaldococcus jannaschii* protein Mj0968 is not a P-type ATPase

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Abstract **The *Methanocaldococcus jannaschii* (formerly *Methanococcus jannaschii*) protein Mj0968 has been reported to represent a soluble P-type ATPase [Ogawa et al., FEBS Lett. 471 (2000) 99–102]. In this study, we report that the heterologously expressed Mj0968-His_{10} protein exhibits high rates of phosphatase activity, whereas only very low ATPase activity was measured. Replacement of the aspartate residue in the DSAGT motif (D7A), which becomes phosphorylated during the reaction cycle of P-type ATPases, does not affect the V_{max}, but only the K_M of the reaction. Labeling studies with [γ-^{32}P]ATP and [α-^{32}P]ATP revealed that the previously reported labeling experiments [Ogawa et al., 2000] do not necessarily show phosphorylation of Mj0968, but rather point to ATP binding. Binding studies with trinitrophenyl adenosine nucleotides showed low apparent K_d values for those molecules. These results provide evidence that the native function of Mj0968 seems to be that of a phosphatase, rather than that of an ATP-hydrolyzing enzyme.**

Key words: Mj0968; P-type ATPase; Phosphorylation; *p*-Nitrophenyl phosphate hydrolysis; Phosphatase; 2′-*O*-Trinitrophenyl nucleotide binding; *Methanocaldococcus jannaschii*

1. Introduction

P-type ATPases are ubiquitous abundant proteins, catalyzing the membrane transport of charged molecules [2]. The protein sequence identity between the different members of the P-type ATPase family is not very high, but they all share some key motifs, which are highly conserved [3] and which suggest a similar architecture of all family members. This was clearly confirmed by elucidation of the structures of the H^+-ATPase, the Na^+,K^+-ATPase, and the Ca^{2+}-ATPase [4–7]. The first high resolution structure of a P-type ATPase, the Ca^{2+}-ATPase (SERCA1a) from rabbit [6,7], provided a detailed picture of how the cytoplasmic loops, i.e. the domains responsible for ATP hydrolysis and phosphorylation, and the transmembrane domains may work together. The catalytic domain, a cytoplasmic loop, of P-type ATPases shares significant similarity with other enzymes. Based on this observation, Aravind et al. [8] proposed the HAD superfamily of enzymes, named after the L-2 haloacid dehalogenase. Proteins of this class contain some conserved key amino acids that are structurally organized to form a binding pocket where the hydrolytic reaction occurs. It was hypothesized [8] that P-type ATPases evolved by fusion of a HAD phosphatase domain with a transmembrane protein, thereby forming an ion transport ATPase. Structural analysis of a HAD phosphatase protein will therefore provide interesting insights into the nucleotide binding and the mechanism of catalysis for the whole enzyme family including P-type ATPases, thereby avoiding the difficulties in structure determination of membrane proteins. In particular, the use of proteins derived from thermophilic organisms may be of great advantage for structure determination, because of the stability of these proteins. A recent example is provided by the MreB structure of *Thermotoga maritima*, an extremely thermophilic bacterium [9].

The open reading frames (ORFs) Mj0968 of *Methanocaldococcus jannaschii* and Mt1493 of *Methanothermobacter thermoautotrophicum* were proposed to encode soluble P-type ATPases, which might represent ancestors of the pro- and eukaryotic ion-transporting P-type ATPases lacking the membrane domain [8]. Furthermore, the genomes of *Methanosarcina acetivorans* and *Methanosarcina matzei* revealed similar ORFs, Ma3999 and Mm0910, respectively. The Mj0968 protein was overproduced and characterized [1]. The authors arrived at the conclusion that the protein is indeed a P-type ATPase judged by ATP hydrolysis, *ortho*-vanadate inhibition of this activity, and the formation of a phosphointermediate. In this report we are able to confirm that Mj0968 is present in the cytosol of *M. jannaschii*, as found by immunoblot analysis and immunoprecipitation. However, our results argue in favor of Mj0968 being a phosphatase, rather than an ATPase.

2. Materials and methods

2.1. Subcloning of the Mj0968 gene and construction of a D7A mutant of Mj0968

The gene Mj0968 was amplified using the polymerase chain reaction (PCR) technique from a commercially available pUC18 plasmid harboring the AMJEP60 fragment of the *M. jannaschii* genomic DNA.

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Abbreviations: TNP-ATP (-ADP, -AMP), 2′- (or 3′-)*O*-(trinitrophenyl)adenosine 5′-triphosphate (di-, monophosphate); pNPP, *p*-nitrophenyl phosphate; TCA, trichloroacetic acid

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The plasmid was purchased from the American Type Culture Collection (ATCC). Forward and reverse oligonucleotide primers (5′-CATATGAAAGTGGCTATAGT-3′ and 5′-CTCGAGCTATTACGAACACTCCTTAATTTGC-3′) for PCR were designed with *Nde*I and *Xho*I restriction sides, respectively.

The aspartate residue in the 7-DSAGT-11 motif was replaced by an alanine using a single step PCR. The used forward primer carrying the altered triplet was 5′-CATATGAAAGTGGCTATAGTGTTT**GCC**AGTGCTGGGACTCTTG-3′. The reverse primer used in this amplification was the same as above. Oligonucleotide primers were purchased from MWG Biotech (Ebersberg, Germany). The PCR products were cloned into a pUC18 vector using the Sure Clone Kit obtained from Amersham-Bioscience (Freiburg, Germany). The ligated pUC derivatives were transformed into competent DH5α cells (Invitrogen, Karlsruhe, Germany). Purified pUC18Mj0968 and pUC18Mj0968-D7A were digested with *Nde*I and *Xho*I. The resulting fragments were ligated into the pET16b vector from Stratagene and transformed in Epicurian Coli® BL21-Codon Plus™-RIL cells (Stratagene, La Jolla, CA, USA) for expression.

2.2. Heterologous expression in Escherichia coli BL21(DE) cells

The constructed pET16bMj0968 and pET16bMj0968-D7A vectors were transformed using calcium chloride-competent Epicurian Coli® BL21-Codon Plus™-RIL cells. Transformants were selected on LB agar plates with chloramphenicol (30 μg/ml) and carbenicillin (50 μg/ml). A single colony was inoculated in liquid rich medium and finally grown in a 5 l fermenter at 37°C. After the cells had reached an optical density at 600 nm of 1.0, expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Two hours after induction the cells were harvested and resuspended in binding buffer (40 mM Tris–HCl, pH 7.8, 10 mM imidazole, 150 mM NaCl, 1 mg/ml deoxyribonuclease). The cell suspension was sonicated five times for 1 min using a sonication probe (micro-tip) and finally passed through a Ribi cell fractionator. The soluble fraction was obtained by ultracentrifugation for 60 min, 100 000×*g* at 4°C.

2.3. Purification of Mj0968-His$_{10}$ and Mj0968-D7A-His$_{10}$

The soluble extract from 5 l induced Epicurian Coli® BL21-Codon Plus™-RIL cells was heated twice up to 80°C for 20 min. Since *M. jannaschii* is a thermophilic Archaeon with a temperature optimum at 85°C [10] the heterologously expressed Mj0968-His$_{10}$ was still soluble, while bacterial proteins denatured. Precipitated proteins were removed by centrifugation at 10 000×*g* for 20 min at 20°C. The supernatant was mixed with 8 ml Ni-NTA slurry obtained from Qiagen (Hilden, Germany), and incubated for 1 h at 4°C in a batch procedure. To minimize the amount of contaminating proteins, the Ni-NTA matrix was overloaded with heat-treated cytosolic extract resulting, however, in losses of recombinant protein in the flowthrough and in the washing fractions. Those fractions were again applied to the affinity matrix. Subsequently, the Ni-NTA material was collected by centrifugation and washed three times with washing buffer (50 mM Tris–HCl, pH 7.8, 60 mM imidazole, 150 mM NaCl). Elution of protein was carried out twice in buffer containing 50 mM Tris–HCl, pH 7.8, 1 M imidazole, 100 mM NaCl. For further analysis the protein was dialyzed against 50 mM HEPES–Tris, pH 7.8, 150 mM NaCl at 25°C.

2.4. Immunoprecipitation

Immunoprecipitation of Mj0968 from *M. jannaschii* cytosolic extract was performed with polyclonal antibodies raised in rabbits against the heterologously expressed Mj0968-His$_{10}$ protein. For the preparation of cytosolic extract of *M. jannaschii*, cells were shock-frozen in liquid nitrogen and cracked in a nitrogen bath using a mortar and pestle. The cell powder was resuspended in 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mg DNase/ml. Unbroken cells were removed by a low speed centrifugation step using an Eppendorf desk centrifuge at 3000×*g* for 10 min at room temperature. Cytosolic extract and membrane fractions were separated by a high speed centrifugation step at 140 000×*g* for 30 min at 20°C. 780 μl cytosolic extract was mixed with 20 μl antiserum and stirred on ice for 2 h. Equilibrated (50 mM Tris–HCl, pH 7.5) protein A-Sepharose CL-4B (250 μl) (Amersham-Bioscience) was added and incubation was continued for 1 h. The Sepharose matrix was pelleted and washed in two volumes of 50 mM Tris–HCl, pH 7.5. Elution of precipitated proteins from protein A was performed in 50 μl 100 mM citric acid, pH 2.5.

2.5. p-Nitrophenyl phosphatase activity

The phosphatase activity of the Mj0968-His$_{10}$ and the Mj0968-D7A-His$_{10}$ proteins was measured using a microtiter assay (Becton Dickinson, Franklin Lakes, MD, USA). The total reaction volume was 100 μl containing 100 mM HEPES–Tris, pH 7.8, 15 mM $MgCl_2$, and 50 μg/ml protein. After preincubation of the mixture at 85°C for 5 min the reaction was started by adding 15 mM *p*-nitrophenyl phosphate (pNPP). The reaction was stopped after 15 min with 100 μl 0.1 M NaOH. The reaction product *p*-nitrophenolate was immediately measured in a microtiter plate reader at 410 nm. The reader was calibrated with a water blank to give the results as true extinction values. The total amount of released *p*-nitrophenolate was calculated with $\varepsilon = 18.5 \times 10^{-3}$ M^{-1} cm^{-1}. Kinetic analysis was carried out using equimolar concentrations of Mg^{2+} and pNPP. Each data point was an average value of duplicates corrected for an average value of duplicates of non-enzymatic reactions. The effect of inhibitors was measured after preincubation for 5 min at 37°C.

2.6. ATPase activity

For determination of the low ATPase activities of recombinant Mj0968-His$_{10}$ and Mj0968-D7A-His$_{10}$, the commercially available EnzCheck® test from Molecular Probes (Eugene, OR, USA) was applied. The reactions were performed according to the manufacturer's protocol with 50 μg of protein in a single measurement with 1 ml reaction volume at 22°C. For ATPase measurements at elevated temperatures (37–85°C) the microtiter plate ATPase assay described by Henkel et al. [11], with modifications given by Altendorf et al. [12], was applied using 50–100 μg of protein/ml for a single measurement. ATP autohydrolysis was subtracted from each data point.

2.7. ATP binding/phosphorylation studies

ATP binding/phosphorylation studies were performed as described [1] with the following modifications: purified Mj0968-His$_{10}$ was incubated in 50 mM HEPES–Tris, pH 7.8, 4 mM $MgCl_2$ in a total volume of 30 μl. After 5 min of preincubation at 56°C, [γ-^{32}P]ATP or [α-^{32}P]ATP was added to a final concentration of 0.33 mM. The reaction was stopped after different time points by addition of 2 volumes chilled 20% trichloroacetic acid (TCA). The denatured proteins were sedimented by centrifugation at 14 000×*g* for 30 min at room temperature. The pellet was neutralized using 4 μl of 1 M Tris, resuspended in sodium dodecyl sulfate (SDS) sample buffer containing 50 mM β-mercaptoethanol and applied to SDS–polyacrylamide gel electrophoresis (PAGE) [13]. The resulting gels were dried and exposed to a phosphorscreen for 24 h at room temperature.

2.8. TNP-adenosine nucleotide binding studies

Fluorescent changes upon 2′- (or 3′-)*O*-trinitrophenyl (TNP)-nucleotide binding to Mj0968-His$_{10}$ were measured at room temperature using an SLM Aminco 8100 spectrofluorometer (SLM Aminco, Rochester, UK). The excitation wavelength was 408 nm and the emission was recorded at 545 nm. The reaction was carried out in 1 ml of 20 mM HEPES–Tris, pH 7.8, 0.5 mM $MgCl_2$, and 2.5 μg of enzyme. The TNP-nucleotides (TNP-ATP, TNP-ADP, TNP-AMP) were titrated from a 1 mM stock solution to obtain concentrations between 0 and 40 μM with a volume increase less than 10%. The measurements were recorded as time trace experiments. After addition of the TNP-nucleotide the total fluorescence was recorded for at least 10 s, in order to determine an average fluorescence. This procedure was more accurate compared with a single point determination, since the fluorescence sometimes oscillated in a small range. The data were plotted after subtraction of the non-enzyme control and standardization. The data were fitted using the equation a [TNP-nucleotide]/b+[TNP-nucleotide] with a representing the maximum fluorescence change and b being the apparent K_d.

The data plots are usually shown in a range up to 25 μM, since the total fluorescence decreases at a certain TNP-nucleotide concentration. This phenomenon is due to quenching effects, which are probably due to an inner filter effect at higher TNP-nucleotide concentrations.

In order to determine the apparent affinity constants (K_d) for ATP, ADP, and AMP, competition experiments with TNP-nucleotides were performed. Therefore, the proteins were preincubated with different concentrations of the desired nucleotide for 5 min at room temperature in 20 mM HEPES–Tris, pH 7.8 and the titration experiments were performed subsequently as described above. For every nucleotide

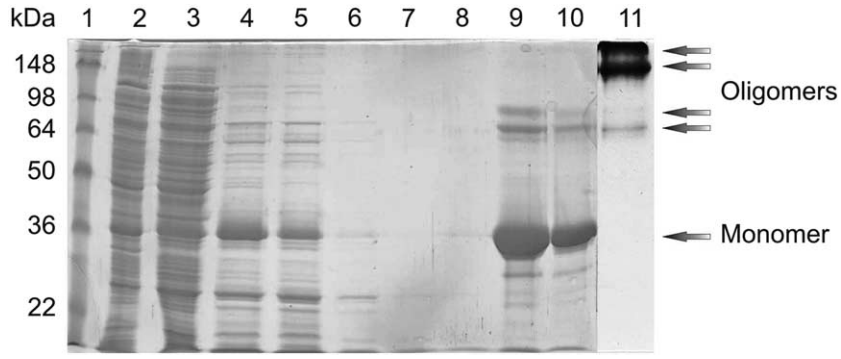

Fig. 1. Purification of the Mj0968-His_{10} fusion protein. Coomassie blue-stained SDS–PAGE of different purification steps. Lane 1, molecular standard in kDa; lane 2, whole cell extract; lane 3, cytosolic extract; lanes 4 and 5, supernatant after precipitation of host cell proteins at 80°C; lanes 6–8, washing fractions from metal affinity chromatography; lane 9, fraction from the first elution; lane 10, fraction from the second elution; lane 11, purified Mj0968-His_{10} without β-mercaptoethanol. Arrows point to the monomeric and oligomeric forms of Mj0968-His_{10}.

concentration, the titration curve was plotted and fitted to determine the apparent affinity constants, which were then plotted against the nucleotide concentrations to obtain the K_d value for the given nucleotide.

2.9. Assays

The presence of the Mj0968-His_{10} protein was tested by immunoblotting [14] using monoclonal pentahistidine antibodies (Qiagen), applying the chemoluminescent method according to the manufacturer's protocol (Perbio Science, Bonn, Germany). SDS–PAGE analysis was carried out according to Laemmli [13].

3. Results

3.1. Cloning, expression and purification of the Mj0968-His_{10} and Mj0968-D7A-His_{10} proteins

Heterologous expression of archaeal genes in a bacterial host like *E. coli* is often limited by the different codon usage of these organisms. To circumvent low synthesis of the Mj0968-His_{10} and Mj0968-D7A-His_{10} proteins in *E. coli*, we used the Epicurian Coli® BL21-Codon Plus™-RIL strain and the pET16b vector. Cells were grown in a 5 l fermenter at 37°C with constant aeration, thereby minimizing the formation of protein aggregates. After cell lysis, the cytosolic extract was heated twice to 80°C (cf. Fig. 1) for the enrichment of the archaeal protein. The protein carrying an N-terminal 10 histidinyl fusion was purified using Ni-NTA chromatography with a protein yield of approximately 40 mg of Mj0968-His_{10} from 5 l culture medium. SDS–PAGE of the different purification steps showed a dominant protein band around 36 kDa, which refers to the monomeric Mj0968-His_{10} protein (Fig. 1). High molecular weight bands were observed, which represent oligomers of Mj0968-His_{10} (as shown by immunoblot analysis, data not shown) and which can be reduced with β-mercaptoethanol to the monomeric form. Small amounts of degradation products of Mj0968-His_{10} can also be seen.

All P-type ATPases contain the well-conserved DKTGT motif located within the phosphorylation domain, in which the aspartate residue becomes phosphorylated during the reaction cycle [19]. To clarify the role of the aspartate residue in the proposed phosphorylation site of the Mj0968 protein (7-DSAGT-11), we substituted the aspartate residue by an alanine residue. The purification procedure for the Mj0968-D7A-His_{10} protein was identical to that of the wild-type protein.

3.2. Immunodetection of Mj0968 in M. jannaschii cells

To determine whether the Mj0968 gene is expressed in vivo in *M. jannaschii*, polyclonal antibodies were raised against the Mj0968-His_{10} protein and used for detection. Immunoblot analysis revealed that the Mj0968 protein was present in the cytoplasmic extract of *M. jannaschii* cells. Furthermore, Mj0968 protein could be immunoprecipitated from the cytosolic extract providing strong evidence that the Mj0968 gene is expressed in vivo (Fig. 2). The oligomeric form (148 kDa) was also observed with the heterologously expressed Mj0968-His_{10} in the absence of β-mercaptoethanol (compare Fig. 1, lane 11).

3.3. Kinetic characterization of Mj0968-His_{10} and Mj0968-D7A-His_{10}

Ogawa et al. [1] determined an ATPase activity (V_{max} 160 nmol P_i/mg/min at 50°C) for the purified Mj0968 protein, which was at least in part inhibited by the phosphate analog *ortho*-vanadate. Surprisingly, the ATPase activity decreased rapidly above 55°C. Since the growth optimum of *M. jannaschii* is around 85°C [10], it was postulated that the protein

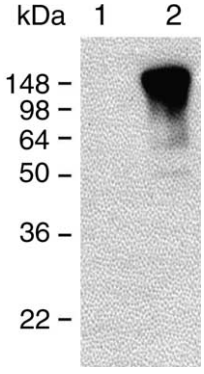

Fig. 2. Immunoprecipitation of Mj0968 from *M. jannaschii* cytosolic extract. *M. jannaschii* cytosolic extract was immunoprecipitated with polyclonal antibodies against the heterologously expressed Mj0968-His_{10}. 10 μl of the elution fraction (lane 2) was separated by SDS–PAGE and Mj0968 was detected by immunoblot analysis. The band represents oligomers of Mj0968 from *M. jannaschii*. Lane 1 shows the protein A-Sepharose CL-4B fraction after elution. The SDS–PAGE was run under non-reducing conditions. It is assumed that as in Fig. 1 the presence of β-mercaptoethanol would result in monomers of Mj0968 being seen on the gel.

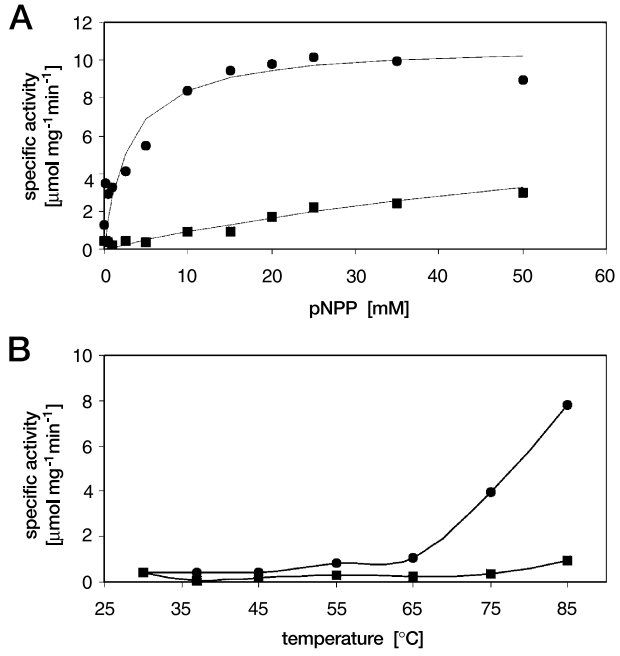

Fig. 3. Phosphatase activity of Mj0968-His_{10}. A Michaelis–Menten plot for the pNPP hydrolysis by Mj0968-His_{10} and Mj0968-D7A-His_{10} mutant protein measured at 85°C is shown in A. 15 mM pNPP and equimolar concentrations of $MgCl_2$ were used. The data were fitted using the Michaelis–Menten equation. The effect of temperature is shown in B. ●, Mj0968-His_{10}; ■, Mj0968-D7A-His_{10}.

needs a membrane-bound counterpart for maintaining the activity at high temperature. In contrast to these findings, we could only measure a very low ATPase activity of 15 nmol/mg/min detectable only by using the sensitive EnzCheck® test from Molecular Probes (data not shown). Since the EnzCheck® assay is a coupled assay it can only be used at room temperature (22°C). Ogawa et al. [1] reported an ATPase activity around 60 nmol/mg/min at 25°C, which is comparable to the activities reported here. Interestingly, the Mj0968-D7A-His_{10} mutant is not affected in its ATPase activity (24 nmol/mg/min, data not shown).

However, we were able to determine a high phosphatase activity measured by the hydrolysis of pNPP. The pNPP hydrolysis increased drastically above 55°C (Fig. 3B). A maximum could not be determined, since the experimental setup

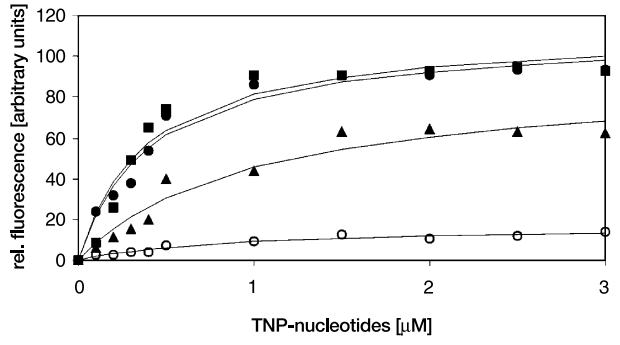

Fig. 4. TNP-adenosine nucleotide binding to Mj0968-His_{10}. Fluorescence changes of TNP-adenosine nucleotides were measured upon binding to Mj0968-His_{10} (2.5 µg). To demonstrate specific binding, protein denatured with 1% SDS was used as a control. ●, TNP-ATP; ■, TNP-ADP; ▲, TNP-AMP; ○, SDS-denatured protein, TNP-ATP.

reached its limit at 85°C. The hydrolytic activity follows classical Michaelis–Menten kinetics (Fig. 3A) with a V_{max} of 10.8 µmol/mg/min and a K_M of 2.9 mM. The pNPP hydrolysis was almost unaffected by *ortho*-vanadate (only 15% inhibition of the pNPPase activity was observed at *ortho*-vanadate concentrations of 2 mM), but was dependent on the presence of Mg^{2+} (data not shown). Interestingly, the Mj0968-D7A-His_{10} derivative still shows phosphatase activity with a similar V_{max} of 9.5 µmol/mg/min, but a drastically increased K_M of 96.5 mM (note that these values are derived from a calculation using the Michaelis–Menten equation, based on the data shown in Fig. 3A).

3.4. TNP-adenosine nucleotide binding to Mj0968-His_{10}

The binding of nucleotides to Mj0968-His_{10} protein was studied using fluorescent nucleotide analogues, such as TNP-ATP. Binding constants for these nucleotides were determined by plotting the relative fluorescence changes against the TNP-nucleotide concentration (Fig. 4). The apparent affinities for TNP-ATP and TNP-ADP are approximately 0.4 µM. For TNP-AMP the apparent affinity is around 1.0 µM. To ensure specificity of the TNP-nucleotide binding, SDS-denatured protein was used as a control.

To determine the correct affinity constants of Mj0968-His_{10} for ATP, ADP, and AMP, displacement studies with TNP-ATP were carried out (Fig. 5A). The apparent K_d values were determined and plotted against the nucleotide concentration in order to determine the K_d value for the nucleotide which was used for preincubation. Since these apparent affinity constants fit a straight line when plotted against the nucleotide

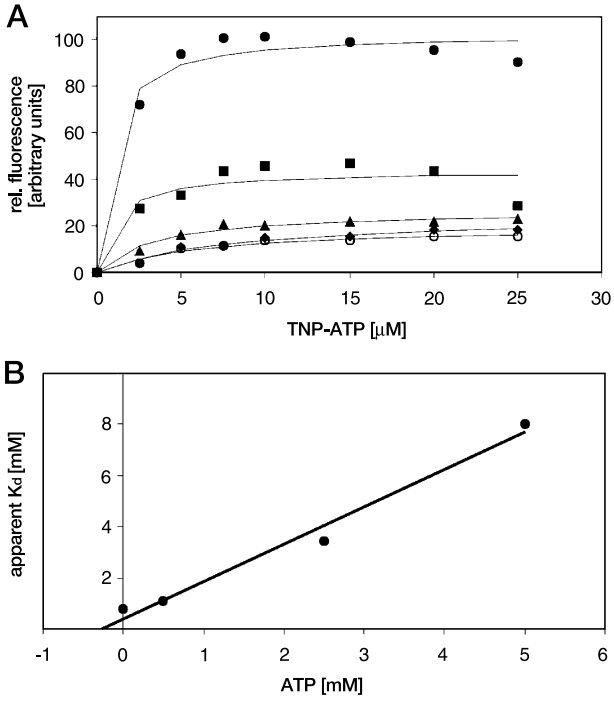

Fig. 5. TNP-ATP displacement studies with Mj0968-His_{10}. In order to determine the affinity constants of adenosine nucleotide binding to the Mj0968-His_{10} protein (2.5 µg), displacement of TNP-ATP was measured. The Mj0968-His_{10} protein (2.5 µg) was preincubated for 5 min at room temperature with ATP. The protein was then titrated with TNP-ATP (A). The calculated apparent K_d values were plotted against the nucleotide concentration (B). ●, 0 mM ATP; ■, 0.5 mM ATP; ▲, 2.5 mM ATP; ♦, 5 mM ATP; ○, 10 mM ATP.

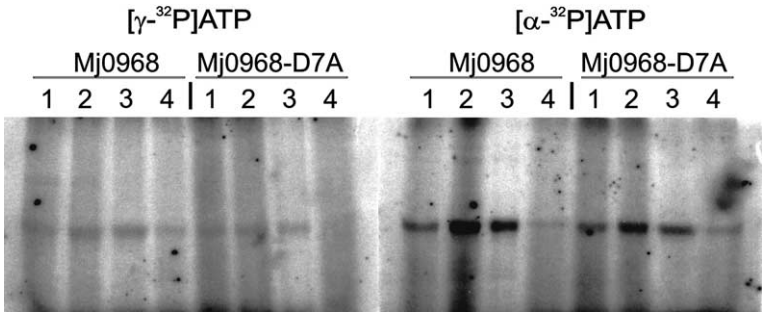

Fig. 6. ATP binding to Mj0968-His_{10} and Mj0968-D7A-His_{10}. Purified proteins (5 μg) were incubated with either 0.33 mM [γ-^{32}P]ATP or [α-^{32}P]ATP at 56°C for 5 min (lane 1), for 20 min (lane 2) or for 20 min after preincubation with 10 mM ATP (lane 3). As a control, TCA-denatured protein was incubated for 20 min (lane 4). The figure shows an autoradiogram of labeled proteins separated by SDS–PAGE.

concentration, the displacement is competitive (Fig. 5B). The dissociation constant was 0.27 mM for ATP (Fig. 5B), 0.76 mM for ADP, and 17.1 mM for AMP.

3.5. *ATP binding to Mj0968-His_{10} and Mj0968-D7A-His_{10}*

In accord with the previously reported results [1], we found for the wild-type Mj0968-His_{10} an increase in the labeling with temperature (data not shown) and time using [γ-^{32}P]ATP (Fig. 6). Interestingly, the Mj0968-D7A-His_{10} mutant protein showed similar results (Fig. 6, left panel). To find out whether ATP might just be trapped in the TCA-denatured protein, we used [α-^{32}P]ATP as a control. Surprisingly, we again detected labeling for both proteins Mj0968-His_{10} and Mj0968-D7A-His_{10} (Fig. 6, right panel). The experimental setup described here does not exclude a covalent modification of Mj0968-His_{10} by ATP. Further experiments will reveal whether Mj0968-His_{10} might be adenylated or if the TCA precipitation traps a non-covalently bound nucleotide within the denatured protein. Binding of ATP to TCA-denatured protein was used as a control.

4. Discussion

It has been reported recently that *M. jannaschii* harbors a soluble P-type ATPase as supported by sequence alignments [8] and biochemical data [1]. Since the existence of such an enzyme would have a profound effect on the discussion about the evolution of P-type ATPases, we set out to investigate the Mj0968 protein in more detail. First, with the help of polyclonal antibodies, we were able to demonstrate that the Mj0968 protein is present in vivo in *M. jannaschii*. This result demonstrates that the corresponding gene is expressed and that the protein itself will certainly fulfill a function in vivo. Second, the Mj0968-His_{10} protein exhibits phosphatase activity as measured by the hydrolysis of pNPP ($K_M \approx 2.9$ mM, $V_{max} \approx 10.8$ μmol/mg/min). The enzymatic activity increased with temperature up to 85°C, which is in accord with the growth optimum of *M. jannaschii*. A similar temperature dependence was recently shown for a membrane-bound P-type ATPase of *M. jannaschii* [20]. The purified Mj1226 P-type ATPase was inactive at 40°C, but the high specific activity, up to 180 μmol/mg/min, was reached at 95°C [20]. Compared to Mj1226, the reported ATPase activity and temperature dependence of Mj0968 seem to be rather obscure [1]. Furthermore, the Mj0968-D7A-His_{10} derivative still exhibits residual phosphatase activity with a similar V_{max} of 9.5 μmol/mg/min, but a drastically increased K_M of 96.5 mM. If Mj0968 was a P-type ATPase, substitution of the aspartate residue in the phosphorylation site would result in a complete loss of phosphatase and ATPase activity. Therefore, we propose that the native function of Mj0968 is not that of an ATPase but rather that of a phosphatase.

The determination of nucleotide binding affinities using TNP-nucleotides for full-length P-type ATPases [15–17,21,22] and separately synthesized modules was reported for several cases [18,23–25]. The K_d value of TNP-ATP for the Na^+,K^+-ATPase cytoplasmic loop was 3.2 μM [18], for the Ca^{2+}-ATPase cytoplasmic loop 1.9 μM [24], for the H^+-ATPase cytoplasmic loop 6.5 μM [23] and 1.89 μM in the case of the Wilson's disease protein cytoplasmic loop [25]. The values obtained for the Mj0968-His_{10} polypeptide are comparable to those determined for the P-type ATPase loops mentioned above.

The specific binding of TNP-nucleotides to Mj0968-His_{10} was measured by the competitive displacement using nucleotides. The resulting affinity constant (K_d) for ATP was 0.27 mM, for ADP 0.76 mM, and for AMP 17.1 mM, indicating the specificity of the binding site. Nevertheless, the low hydrolytic activity and the decrease of this activity above 55°C [1] do not argue in favor of an in vivo ATPase activity. Furthermore, Mj0968-His_{10} does not necessarily form a phosphointermediate. The experiments performed with the Mj0968-D7A-His_{10} mutant protein and the use of [α-^{32}P]ATP lend support to the notion that the labeling experiments measure ATP binding, but not the formation of a phosphointermediate. All these results argue against the notion that Mj0968 represents a soluble P-type ATPase or is part of a kind of composite ATPase. Rather, the tight ATP binding argues in favor of a regulatory role for ATP.

That the Mj0968 protein acts together with a membrane-bound counterpart as an ion transport ATPase is also highly unlikely, since the membrane part of P-type ATPases is interrupted by two cytoplasmic domains of which only the larger one is structurally homologous to Mj0968. Therefore, we suggest that Mj0968 is a soluble phosphatase in the *M. jannaschii* cytosol.

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