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Expression and characterization of a *Synechocystis* PCC 6803 P-type ATPase in *E. coli* plasma membranes

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Abstract

In a previous paper, we published the sequence of a P-type ATPase gene from *Synechocystis* 6803 [Geisler et al. (1993) J. Mol. Biol. 234, 1284] which showed significant homologies to eukaryotic calcium ATPases. To investigate the specificity and activities of this plasma membrane-bound enzyme, we expressed the slightly modified gene in an ATPase deficient *E. coli* strain. The expressed ATPase showed an apparent molecular mass of about 97 kDa and is localized in the *E. coli* plasma membranes. The introduced 6xHis tag at the N-terminus allowed the purification of the *Synechocystis* 6xHis-ATPase by single-step affinity chromatography using a Ni^{2+}-nitrilotriacetic acid resin. The ATPase activity of the enzyme is inhibited by vanadate ($IC_{50} = 119\,\mu M$), *N*-ethylmaleimide, *N,N*-dicyclohexylcarbodiimide, and inhibitors of eukaryotic sarco(endo)plasmic reticulum Ca^{2+}-ATPases; however, it is stimulated by thapsigargin. Formation of phosphorylated enzyme intermediates depends on calcium ions indicating that the *Synechocystis* P-ATPase acts as a calcium pump equivalent to eukaryotic sarco(endo)plasmic reticulum Ca^{2+}-ATPases. © 1998 Elsevier Science B.V.

Keywords: P-ATPase; Calcium; Heterologous expression; Ni-chelate chromatography; (Cyanobacteria); (*Synechocystis* PCC 6803)

1. Introduction

P-type ATPases are membrane-bound enzymes of about 70–120 kDa which catalyze the transport of various cations [1] or aminophospholipids [2]. They possess regions of high sequence homologies; the catalytical process includes an unstable phospho-enzyme (EP). P-ATPases are not related to the F-ATPases of energy-conserving membranes in chloroplasts, mitochondria, and bacteria [3]. While F-ATPases are well-known in cyanobacterial membranes [4], the investigation of P-type ATPases is more difficult.

In the last years, an increasing number of P-

Abbreviations: BHQ, 2,5-di(tert-butyl)-1,4-benzohydroquinone; CPA, cyclo-piazonic acid; CPM, cytoplasmic membrane(s); DCCD, *N,N'*-dicyclohexylcarbodiimide; EP, phosphoenzyme; IPTG, isopropyl-ß-D-thiogalactoside; NEM, *N*-ethylmaleimide; Ni-NTA, Ni^{2+}-nitrilotriacetic acid; PM, plasma membrane; PMCA, plasma membrane Ca^{2+}-ATPase(s); SER, sarco(endo)plasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase(s); TG, thapsigargin

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ATPases of different specificity were found in cyanobacterial strains [5–11]. Two genes of strain *Synechococcus* PCC7942 are likely to code for copper ATPases (pacS [9] and ctaA [11]). The gene product of the third gene, pacL [8], is bound to cytoplasmic membranes and is likely to be a Ca^{2+}-ATPase [5]. Ca^{2+}-ATPases were also proposed for the cyanobacterial strains *Anabaena variabilis* [10] and *Synechocystis* PCC 6803 [12]. Recently, the analysis of the entire genome of *Synechocystis* 6803 [13] has revealed the existence of nine P-ATPases genes. Besides the one described here, three more putative Ca^{2+}-ATPase genes were identified. The other five ATPase genes resemble typical prokaryotic P-ATPases; two of them show striking homologies to cadmium pumps, and one to the kdpB subunit of the *E. coli* potassium pump [14].

The *Synechocystis* PCC 6803 P-ATPase described in [12] is cotranscribed with a GTPase gene located downstream from the ATPase gene [15]. The ATPase is closely related to the *Synechococcus* 7942 Ca^{2+}-ATPase [5]. Features like molecular mass, sequence homology, transmembrane organization and homologies in the proposed calcium-binding domain are remarkably similar to eukaryotic Ca^{2+}-ATPases of the SER-type [12]. These SERCA [16–18] differ from the Ca^{2+}-ATPases of the PM-type [19–21] in a number of biochemical and functional properties.

In this report, we characterize the P-ATPase of *Synechocystis* PCC 6803. An efficient expression system of the 6xHis tagged ATPase was established in *E. coli*; both isolated and membrane-bound enzymes were studied. Results on phosphoenzyme formation, ATPase activity and sensitivity to ATPase inhibitors point to a calcium specificity of the enzyme.

2. Materials and methods

2.1. Materials

ATP and *n*-octylglucoside (obtained from Boehringer-Mannheim), (gamma-^{32}P)ATP (Amersham), thapsigargin and BHQ (Calbiochem), DCCD, cyclopiazonic acid (CPA), bovine calmoduline and sodium orthovanadate (Sigma and Alfa Produkte-Karlsruhe), and other chemicals were of the highest purity available.

2.2. Oligopeptide synthesis and antiserum production

A 15-mer oligopeptide (residues 651–664 of the *Synechocystis* enzyme [12]) was synthezised, coupled to keyhole limpet hemocyanin and used to immunize rabbits s.c. with 500 μg of coupled peptide. Antisera against the whole enzyme were raised by application of 100 μg of *Synechocystis* 6xHis ATPase protein purified by metal chromatography. Injections were repeated after 3 weeks in Freund's incomplete adjuvans. Antisera were purified by caprylic acid and ammonium sulfate precipitation.

2.3. Expression of the Synechocystis 6803 P-ATPase in E. coli

Due to a stable secondary structure near the start codon of the P-ATPase mRNA, direct expression of the protein in *E. coli* failed. Using the vector encoded 6xHis tag, the secondary structure was cut out and six histidines were inserted at the N-terminus of the P-ATPase. The first 5 aa of the native enzyme (MDFPT) were, therefore, substituted by the sequence MRGSHHHHHHGIRMRARYP. The 4.7 kb *Hin*dIII fragment containing the *Synechocystis* P-ATPase gene [12] was restricted with *Afl*II, blunt-ended with Klenow enzyme and restricted with *Hin*dIII. The resulting 3.8 kb fragment was ligated in frame into the multi-cloning region of the expression vector pQE32 (Qiagen, Diagen GmbH, Hilden, Germany) precut with *Sma*I and *Hin*dIII.

The engineered plasmid pQE32-8 was used to transform the *E. coli* strain TKR2000 [14] carrying the repressor plasmid pREP4 (Qiagen). Large-scale expression cultures (400 or 800 ml SB (SB: 25 g bacto-tryptone, 15 g yeast extract, 10 g KCl per liter)) supplemented with 2% glucose (w/v), ampicillin (200 μg/ml) and kanamycin (25 μg/ml) were inoculated 1:50 with mid-log precultures. At a cell density of 0.4 (550 nm), 10 μM IPTG was added; after 3–4 h, cells were harvested by centrifugation, washed with washing buffer (1 mM EDTA, 50 mM Tris/Cl pH 8.0) and stored frozen.

2.4. Purification of the expressed Synechocystis 6xHis-ATPase

All steps were performed at 4°C and all media contained the protease inhibitor pefa-block (50 μM;

Boehringer). Cells were resuspended in glycerol buffer (5 ml/g wet weight; 300 mM NaCl, 30% (v/v) glycerol, 1 mM ß-mercaptoethanol, 10 mM Tris/Cl pH 8.0, and *n*-octylglucoside (46 mM) was added. After freezing in N_2 and thawing on ice, cells were sonicated; the expressed 6xHis-ATPase was solubilized by stirring for 1 h at 0°C. After addition of Ni-NTA agarose (5 ml/g wet weight; Qiagen) and 1 h agitation, the resin was packed into a column. For analytical chromatography, the column was washed with glycerol buffer containing 0–40 mM imidazole and 4.6 mM *n*-octylglucoside until the A280 of the flow-through was less than 0.01. The 6xHis-ATPase was eluted by a FPLC-mediated 0–500 mM imidazole gradient containing 0.46 mM *n*-octylglucoside. For preparative chromatographies, a third washing step with 60 mM imidazole plus 4.6 mM *n*-octylglucoside was added and the protein was eluted by 5–10 ml of glycerol buffer containing 250 mM imidazole plus 0.46 mM *n*-octylglucoside. Immuno-positive fractions were pooled and frozen in aliquots.

2.5. Isolation of recombinant E. coli plasma membranes

After lysis in washing buffer and removal of cellular debris, recombinant plasma membranes of *E. coli* TKR2000/pQE32-8 were pelleted by centrifugation at $100\,000 \times g$ for 60 min and washed with 50 mM Tris/Cl pH 8.0. Membrane vesicles were homogenized in small volumes of glycerol buffer through syringes. Aliquots were stored at a concentration of 2.5 μg/μl at −70°C until use.

2.6. Phosphorylated intermediates of the Synechocystis Ca^{2+}-ATPase

Phosphoenzyme formation with (gamma-^{32}P)ATP was carried out in 50 μl of ice-cold medium containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, and the specified supplements for 20 s [22]. *Synechocystis* cytoplasmic membranes isolated by saccharose-gradient centrifugation, recombinant plasma membranes from *E. coli* TKR2000/pQE32-8 (10–50 μg protein), or purified *Synechocystis* 6xHis-ATPase (2 μg protein) were added. After 10 min, phosphorylation was terminated by the addition of 0.5 ml 10% TCA. After 15 min at 0°C, the pellets were washed once with ice-cold distilled water. For SDS gel electrophoresis, samples were resuspended in 10 μl of Papp loading buffer [23] for 10 min at room temperature using a Hamilton syringe. For hydroxylamine treatment, aliquots were incubated with 50 mM hydroxylamine at 0°C for 10 min. Phosphorylated membranes were separated by 7.5% PAGE of the Laemmli type [24]; phosphorylated intermediates of the purified 6xHis-ATPase were run on acidic 7.5% SDS-polyacrylamide gels of the Sarkadi type [18] at 4°C. Gels were Coomassie stained, dried and autoradiographed.

2.7. ATP hydrolysis assays

ATPase activity of the membrane-bound (plasma membrane vesicles from *E. coli* TKR2000/pQE32-8 (30–100 μg of protein)) or purified 6xHis-ATPase (2–7.5 μg of protein) was measured at 37°C in a volume of 1 ml. After preincubation for 10 min in hydrolysis buffer (50 mM KCl, 50 mM $MgCl_2$, 50 mM HEPES/KOH pH 7.5) with the specified supplements, the reaction was started by the addition of 2 mM (gamma-^{32}P)ATP. Aliquots of 300 μl were drawn after 5, 10 and 15 min, stopped with 1 M $HClO_4$, and radioactivity of inorganic phosphate was measured by liquid scintillation counting. Plasma membrane vesicles from *E. coli* TKR2000/pQE32 (without ATPase insert) show ATP hydrolysis rates like the blank values (no enzyme addition); the experimental data were corrected by these blank values.

3. Results

3.1. Expression and purification of the Synechocystis 6xHis-ATPase by Ni-chelate chromatography

Overexpression of membrane proteins in *E. coli* normally causes toxic effects on host cells [25,26]. Over-production must therefore controlled strictly. We used the expression vector pQE32 in combination with the repressor plasmid pREP4 (producing high levels of lac repressor). As *E. coli* itself has a plasma membrane-bound K^+-ATPase of the P-type which might interfere with our physiological measurements (kdpB, [14]), we used the genetically engineered strain of *E. coli* ''TKR2000'' lacking the ATPase

activities of both the kdp (deltakdpABCDE81) and unc (deltaIBEFHA) system [14]. Mild induction of *E. coli* TKR2000/pQE32-8 with 10 μM IPTG led to the expression of a 97 kDa immunopositive band in total cell extracts (Fig. 1(A) lane 1). After 3 h, expression was maximal (lane 3); smaller peptides in this preparation may represent truncated expression products. In membrane preparations of induced *E. coli* TKR2000/pQE32-8 cells, most of the recombinant ATPase could be immunodetected in the membrane fractions (Fig. 1(B), lane P2); only a small amount was found in the washing medium (lane S1 and S2). The yield of membrane preparations was up to 5 mg of total plasma membrane protein from 1 g

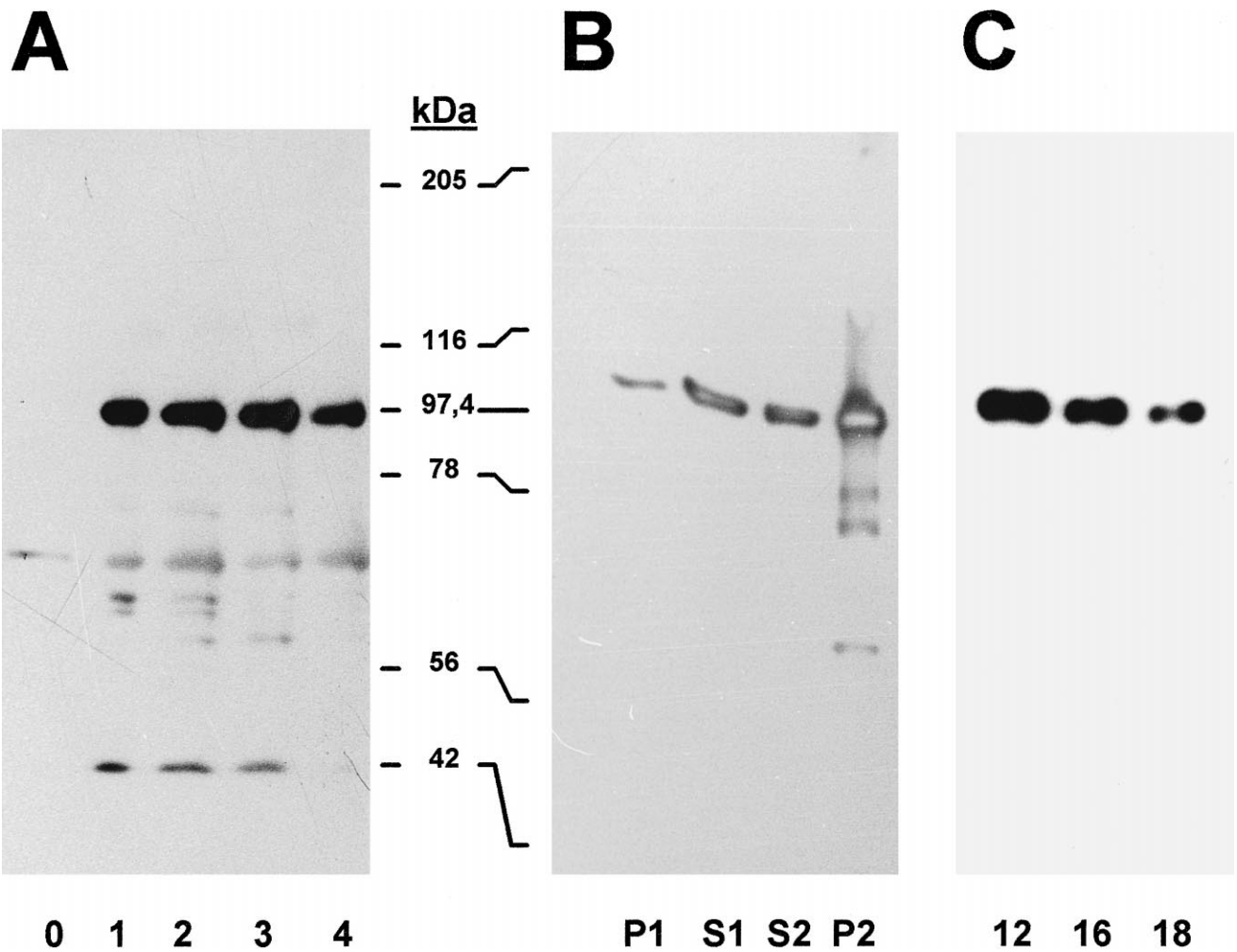

Fig. 1. Expression of the *Synechocystis* Ca^{2+}-ATPase in *E. coli*. (A) *E. coli* TKR2000/pQE32-8 was grown in SB medium, and total cell extracts were taken before (lane 0), and 1, 2, 3, and 4 h (lane 1–4) after induction with 10 μM IPTG. (B) Cells were harvested after 3 h, lysed by sonication and cellular debris was removed by centrifugation at 10 000 × *g* (P1). The supernatant (S1) was centrifuged at 100 000 × *g* and the pellet was resuspended and washed by centrifugation as before (supernatant = S2; pellet = P2). (C) Sonicated cells were treated with 46 mM *n*-octylglucoside; the extract was bound to Ni-NTA agarose by stirring at 0°C for 1 h. The agarose was washed with glycerol buffer containing 0, 20 and 40 mM imidazole in the batch, packed into the column and eluted by a linear FPLC-mediated gradient (0–500 mM imidazole containing 0.46 mM *n*-octylglucoside). Aliquots of fractions 12, 16 and 18 were taken for separation. All samples were subjected to 7.5% PAGE, blotted onto nitrocellulose and immunostained with antisera raised against the synthesized peptide (see Section 2).

(wet weight) of cells. Quantitation of the ATPase by Western analysis using isolated 6xHis-ATPase as a standard revealed that the content of the *Synechocystis* ATPase on the total plasma membrane protein was between 20 and 25%.

The 6xHis tag allowed rapid purification of the expressed P-ATPase [27]. Therefore, the expressed 6xHis-ATPase was extracted from *E. coli* plasma membranes by solubilization using the non-ionic detergent *n*-octylglucoside and loaded to Ni-NTA agarose. Using a FPLC-mediated 0–500 mM imidazole gradient, most of the 6xHis-ATPase is eluted between 70 and 200 mM. For large-scale preparations, a step gradient was applied with a third extensive washing step containing 60 mM imidazole; up to 300 μg of highly purified ATPase protein (from 1 g (wet weight) of cells) were eluted with 250 mM (Fig. 1(C)). Under denaturing conditions (8 M urea/1% TX-100) the yield was threefold higher; we assume that the 6xHis tag in the native conformation binds less efficiently to the Ni-NTA matrix. This preparation was dialyzed and directly used for the production of highly sensitive polyvalent antisera. With those, the P-type ATPase was exclusively detected in cyto-

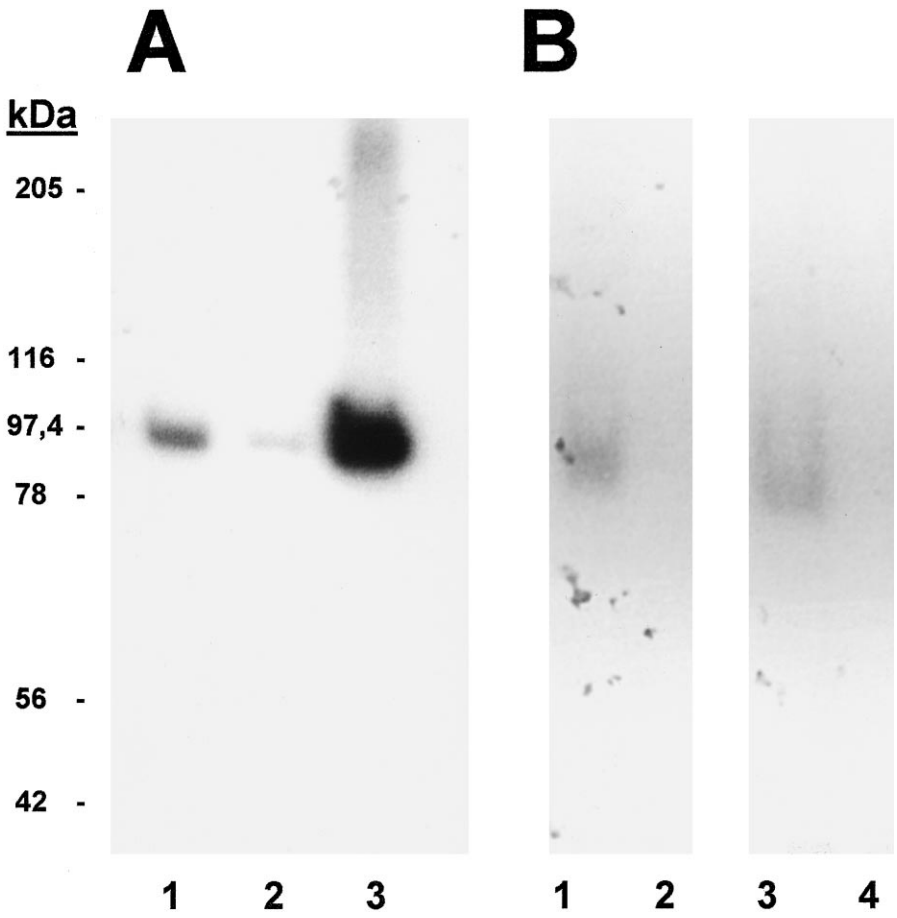

Fig. 2. Phosphorylated intermediates of isolated and membrane-bound *Synechocystis* Ca^{2+}-ATPase. (A) 2 μg of the isolated enzyme expressed in *E. coli* and purified by Ni-chelate chromatography were incubated with (gamma-^{32}P)ATP in MOPS/KCl/$MgCl_2$/500 μM Ca^{2+} medium (lane 1) supplemented with either 5 mM EGTA (lane 2) or 100 μM La^{3+} (lane 3). (B) Recombinant plasma membranes from *E. coli* TKR2000/pQE32-8 (10 μg protein, lanes 1 and 2) or *Synechocystis* cytoplasmic membranes isolated by saccharose-gradient centrifugation (10 μg protein, lanes 3 and 4) were incubated with (gamma-^{32}P)ATP in the presence of 100 μM Ca^{2+}. Aliquots were treated with hydroxylamine (lanes 2 and 4). Phosphorylated intermediates of the isolated and membrane-bound enzyme were separated on 7.5% PAGE of the Sarkadi or Laemmli type, respectively, as described in Section 2.

plasmic membrane fractions of *Synechocystis* 6803 isolated by saccharose-gradient centrifugation (results not shown) verifying previously published results [4].

3.2. Phosphoenzyme formation of the Synechocystis 6xHis-ATPase

Upon addition of Mg-ATP, P-ATPases form a phosphorylated enzyme intermediate (E_1P; [16]). Therefore, the expressed, isolated enzyme was exposed to (gamma-^{32}P)ATP under different conditions. Acidic SDS-PAGE of the Sarkadi type [18] showed that indeed a phospho-intermediate is formed (Fig. 2(A), lane 1) and that it is highly sensitive to higher temperatures (results not shown) and to hydroxylamine (Fig. 2(B), lanes 2 and 4), indicating an acylphosphate linkage. Phosphoenzyme formation is calcium dependent; chelation of calcium by EGTA (5 mM) reduces phosphorylation strongly (Fig. 2(A), lane 2). The signal is strongly enhanced by the addition of La^{3+} (Fig. 2(A), lane 3), an agent which blocks the reaction $E_1P \rightarrow E + P$ in the phosphorylation/dephosphorylation equilibrium thus increasing the steady state concentration of phosphorylated enzyme intermediate [18]. Recombinant plasma membranes from *E. coli* TKR2000/pQE32-8 and cytoplasmic membrane fractions from *Synechocystis* 6803 gave similar results (Fig. 2(B)) verifying both the immunologically determined size and intracellular localization of the enzyme.

3.3. ATP hydrolysis by the Synechocystis 6xHis ATPase

Both preparations of 6xHis-ATPase – isolated and membrane-bound – hydrolyze ATP. pH and temperature dependence of the *Synechocystis* ATPase activity show broad optima (Fig. 3) with an alkaline pH optimum similar to the SERCA3 isoform [28]. In the presence of calcium, the ATPase activity of membrane vesicles show a linear rate of $3.16 \pm 0.30\ \mu mol/h \times mg$ of protein; the rates of the isolated enzyme are about ten times higher. Considering an inside–out orientation of membrane-vesicles of about 50% and an ATPase proportion of 20%–25% on total membrane protein, the ATPase activities of isolated and of membrane-bound enzymes are, therefore, equivalent.

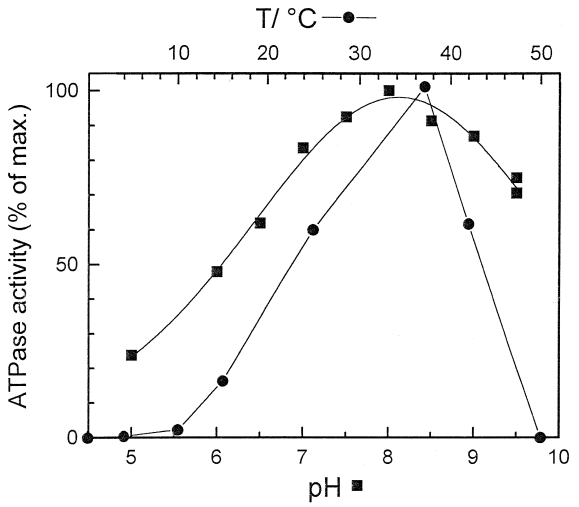

Fig. 3. pH and temperature dependence of ATPase activity of the expressed *Synechocystis* ATPase. ATPase activity of recombinant plasma membranes from *E. coli* TKR2000/pQE32-8 (50 μg protein) was assayed at 37°C at various pH values (squares). Temperature dependent ATP hydrolysis was determined at pH 7.5 using 2 μg of isolated enzyme in hydrolysis buffer plus 100 μM Ca^{2+} (circles). Rates are calculated in percent of the maximum activity.

A strong Ca^{2+}-stimulation was found in the EP formation experiments (Fig. 2(A)); however, calcium effects on ATPase activities are low. Addition of EGTA (5 mM) instead of $CaCl_2$ reduced the rate of hydrolysis by the membrane-bound enzyme from 3.2–2.9 μmol/mg × h. Addition of the calcium ionophore A23187 in the presence of calcium gave rates of 3.7 μmol/mg × h; the overall stimulation by calcium is, therefore, about 25%–30% for the membrane-bound enzyme.

Determination of the ATPase activity showed a sensitivity to micromolar concentrations of vanadate with an IC_{50} of 119 μM. Total inhibition of the enzyme is obtained by millimolar concentrations (Fig. 4). The effect of various inhibitors on ATP hydrolysis of both the membrane-bound and isolated *Synechocystis* enzyme is summarized in Table 1. The ATPase activity was not affected (inhibition $< 10\%$) by 1 mM nitrate, 5 mM NEM and 100 μM bafilomycin A_1, efficient inhibitors of V-ATPases [29]. DCCD inhibited the ATPase activity up to 40%. The inhibition by NBD-Cl (about 30%) might be explained by binding to the conserved Tyr475 [12] as part of the motif YVKGAP which is thought to be

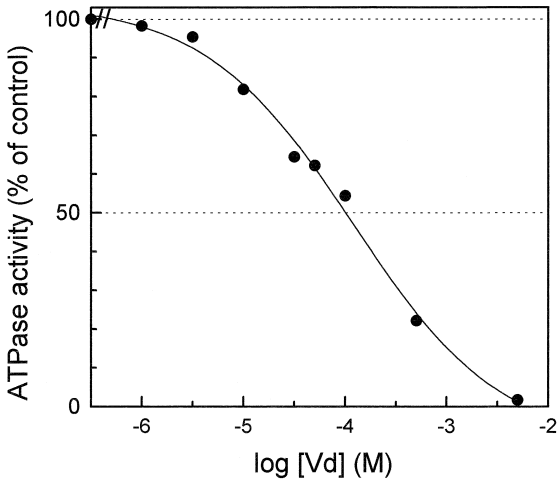

Fig. 4. Vanadate sensitivity of ATPase activity of the expressed *Synechocystis* ATPase. Plasma membrane vesicles from *E. coli* TKR2000/pQE32-8 (37.5–50 μg Protein) were preincubated for 10 min in hydrolysis buffer plus 100 μM Ca^{2+} at 37°C, together with varying concentrations of vanadate, and rates of ATP hydrolysis were determined after addition of (gamma-^{32}P)ATP. Control activitiy was 3.61 μmol/h × mg protein, and inhibition is given in percentage of control rate. The data were fitted in a sigmoidal Boltzman curve; 50% inhibition was at 119 μM vanadate.

Table 1
Effect of various inhibitors on the ATPase activity of the *Synechocystis* 6803 6xHis-ATPase expressed in *E. coli*

Inhibition of ATPase activity (% of control)			
Inhibitor	Conc. [μM]	Isolated enzyme	Membrane-bound enzyme
Vanadate	100	41	51
Cyclopiazonic acid	25	80	81
	100	60	n.d.
BHQ	100	91	99
	1000	72	n.d.
Erythrosin B	1	84	77
Thapsigargin	100	132	154
	250	n.d.	168
DCCD	100	68	60
NBD-Cl	40	n.d.	69

Recombinant plasma membranes from *E. coli* TKR2000/pQE32-8 or the 6xHis-ATPase purified by Ni-chelate chromatography were preincubated in the presence of inhibitors in hydrolysis buffer for 10 min. ATP hydrolysis rates were determined as described in Fig. 1.
n.d., Not determined.

part of the ATP-binding region [17]. 1 μM erythrosin B, an inhibitor of both SER and PM Ca^{2+}-ATPases, showed 23% inhibition. CPA, BHQ and thapsigargin, specific inhibitors of SER Ca^{2+}-ATPases in animal cells, have various effects on the *Synechocystis* P-ATPase: The highest concentration of CPA tested (100 μM) gave about 40% inhibition, 28% inhibition of the isolated enzyme was reached by 1 mM BHQ. An interesting effect showed micromolar concentrations of the sesquiterpene thapsigargin [17,23]; this inhibitor stimulated the ATPase activity of the *Synechocystis* P-ATPase in independent experiments up to 80%.

4. Discussion

Investigations of cytoplasmic membranes from *Synechocystis* 6803 – either physiologically or immunologically – have shown that the P-type ATPase is present in very small amounts (unpublished data). To prove the assumed calcium specificity of this P-ATPase, we, therefore, developed a heterologous gene expression system in order to characterize this cyanobacterial enzyme. Despite of its toxicity in prokaryotic systems, our results demonstrate that *E. coli* can be used for the expression of a functional P-ATPase if maximum repression and mild induction is employed (see also [26]). As described for other P-ATPase expression systems [21,25], the *Synechocystis* ATPase is targeted to the plasma membranes of the ATPase deficient mutant strain *E. coli* TKR2000 [14]; this strain offers the easy investigation of the membrane-bound enzyme without reconstitution. A prerequisite of membrane integration might be the pentapeptide motif R/KILLL in the first transmembrane domaine which is thought to be responsible for ER targeting of Ca^{2+}-ATPases of the SER- but not of the PM-type [30]; this motif is also conserved in the *Synechocystis* sequence (aa 80–84).

A second advantage of the expression system reported here lies in the efficient purification protocol of the expressed 6xHis-ATPase by Ni-chelate affinity chromatography [27]. The ATPase protein can be purified to homogeneity by a single chromatography step. So far, we have no indications for negative effects of the 6xHis tag on the expressed enzyme.

The *Synechocystis* ATPase expressed in *E. coli* is able to form phosphoenzyme intermediates and to hydrolyze ATP both membrane embedded and in purified form. Our data support the assumption that the enzyme acts as a Ca^{2+}-ATPase in vivo: In the presence of (gamma-^{32}P)ATP, the ATPase forms a hydroxylamine sensitive 97 kDa phosphointermediate in a strongly calcium dependent manner. The phosphointermediate is stabilized by the trivalent lanthanum cation as described for mammalian PM Ca^{2+}-ATPases [18] and the SERCA3 isoform of rat kidney [23].

In contrast to the calcium effect on EP formation, stimulation of the ATPase activity of the *Synechocystis* enzyme by calcium was low. Similar results have been reported for a microsomal Ca^{2+}-ATPase of yeast [31] and for the ER-localized Ca^{2+}-ATPase of carrot cells [32]; in these systems, a high basal Mg^{2+} ATPase activity and an inefficient coupling of ATP hydrolysis and calcium transport have been suggested. On the other hand, addition of the calcium ionophore A23187 stimulated the ATPase activity of our membrane fractions.

The *Synechocystis* ATPase activity is inhibited by micromolar concentrations of vanadate; the apparent vanadate affinity of the calcium pump is about 119 μM. This value is in the same range as found for SER Ca^{2+}-ATPase isoforms [28,31], but is much higher than IC_{50} values for PM Ca^{2+}-ATPases (2–3 μM; [19]). The Ca^{2+}-ATPase from *Synechococcus* 7942 seems to be more sensitive to vanadate; its calcium transport is totally blocked by 250 μM vanadate [5]. The *Synechococcus* enzyme is more closely related to the *Synechocystis* 6803 ATPases sll062 and sll0822 (following the nomenclature of Kaneko et al. [13]); one might therefore speculate that the vanadate sensitivities of these *Synechocystis* enzymes are higher than of our enzyme.

Specific inhibitors of Ca^{2+}-ATPases like erythrosin B, CPA and BHQ [33,34] also inhibited the *Synechocystis* ATPase. Calmodulin (CaM), the regulatory protein of eukaryotic PM-type calcium pumps had no effect on the ATPase activity of the *Synechocystis* ATPase (results not shown). In agreement, sequence alignment analysis of the highly conserved CaM-binding domain located near the C-terminus of PMCA isoforms [35] reveals that this domain is absent in the *Synechocystis* pump. The CaM-binding domain is also lacking in the Ca^{2+}-ATPases of *Synechococcus* 7942 [5,8].

Up to now, little is known about prokaryotic calcium pumps [1,36] although they are more common than previously thought. Assuming a calcium specificity of the enzyme, the data presented here show that the *Synechocystis* enzyme is more closely related to SER Ca^{2+}-ATPases [16,28] than to PM calcium pumps [19,20]. Most of the properties of the *Synechocystis* enzyme – like molecular mass, sequence homology, sensitivity to vanadate and SERCA inhibitors – are in line with features described for eukaryotic Ca^{2+}-ATPases of the SER-type [16,18,28].

An exception and an interesting effect which is currently under investigation showed thapsigargin, a highly specific inhibitor of all tested ER Ca^{2+} transport ATPases [17]. It stimulates the ATPase activity of the *Synechocystis* P-ATPase in micromolar concentrations showing a specific interaction of the ATPase with this plant-derived sesquiterpene. By chimeric fusion experiments, the third transmembrane segment M3 has been shown to form the major binding region for thapsigargin [37].

Further characterization of the *Synechocystis* P-ATPase toward its putative calcium specificity, and analysis of other prokaryotic (and especially cyanobacterial) calcium pumps are in progress and will lead to a better understanding of the role of prokaryotic Ca^{2+}-ATPases.

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