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Large-scale purification of the proton pumping pyrophosphatase from *Thermotoga maritima*: A "Hot-Solve" method for isolation of recombinant thermophilic membrane proteins

Rosa L. López-Marqués [a,b,*,1], José R. Pérez-Castiñeira [a], Morten J. Buch-Pedersen [b], Sergio Marco [c], Jean-Louis Rigaud [c], Michael G. Palmgren [b], Aurelio Serrano [a,*]

[a] *Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla, CSIC, Avda. Americo Vespucio 49, 45092 Sevilla, Spain*
[b] *Department of Plant Biology, The Royal Veterinary and Agricultural University (KVL), Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark*
[c] *Institut Curie, UMR-CNRS 168 and LRC-CEA 34V, 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France*

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Abstract

Although several proton-pumping pyrophosphatases (H^+-PPases) have been overexpressed in heterologous systems, purification of these recombinant integral membrane proteins in large amounts in order to study their structure–function relationships has proven to be a very difficult task. In this study we report a new method for large-scale production of pure and stable thermophilic H^+-PPase from *Thermotoga maritima*. Following overexpression in yeast, a "Hot-Solve" procedure based on high-temperature solubilization and metal-affinity chromatography was used to obtain a highly purified detergent-solubilized TVP fraction with a yield around 1.5 mg of protein per litre of yeast culture. Electron microscopy showed the monodispersity of the purified protein and single particle analysis provided the first direct evidence of a dimeric structure for H^+-PPases. We propose that the method developed could be useful for large-scale purification of other recombinant thermophilic membrane proteins.

Keywords: H^+-PPase; *Thermotoga maritima*; Ni-NTA affinity chromatography; Detergent solubilization; Single particle analysis

1. Introduction

Membrane-bound proton-translocating inorganic pyrophosphatases (H^+-PPases, EC 3.6.1.1) are integral membrane proteins that couple pyrophosphate (PPi) hydrolysis to H^+ movement across biological membranes [1]. H^+-PPases have been shown to occur in higher plants, parasitic protists, photosynthetic and thermophilic bacteria, archaea [2] and, more recently, in eggs and ovaries of the insect *Rhodnius prolixus* [3]. Several H^+-PPases have been isolated from the native membranes [4], but due to their low levels of expression the yield of purified protein has been very low. Heterologous expression in *E. coli* or *S. cerevisiae* has been repeatedly used as a tool for biochemical characterization, as both organisms lack an endogenous H^+-PPase [4–7]. Nevertheless, to our knowledge, no successful purification procedure has been reported so far for isolation of recombinant H^+-PPases. Indeed, integral membrane proteins pose particular challenges for

Abbreviations: PPi, inorganic pyrophosphate; H^+-PPase, proton-pumping pyrophosphatase; DTT, ditiothreitol; DDM, n-dodecyl-β-D-maltopyranoside; TVP, *Thermotoga maritima* membrane-bound H^+-PPase; PMSF, phenyl-methyl-sulphonyl-fluoride; MES, 2-morpholinoethansulfonic acid; NTA, nitriloacetic acid; Tris, Tris-(hydroxylmethyl) aminomethane; EM, electron microscopy; SOM, self-organizing mapping

* Corresponding authors. R.L. López-Marqués is to be contacted at Department of Plant Biology, The Royal Veterinary and Agricultural University (KVL), Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark. Tel.: +45 3528 2595; fax: +45 3528 3365. A. Serrano, tel.: +34 954489524; fax: +34 4460065.
E-mail addresses: rlo@kvl.dk (R.L. López-Marqués), aurelio@cica.es (A. Serrano).
[1] Current address: Department of Plant Biology, The Royal Veterinary and Agricultural University (KVL), Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

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expression, purification and structural characterization, and their thermophilic counterparts are becoming the proteins of choice due to their higher stability. Still a high-throughput structural genomics project currently in process for the hyperthermophilic bacterium *Thermotoga maritima* failed to systematically express and purify integral membrane proteins [8]. Therefore, development and/or improvement of systems for the heterologous expression and isolation of large amounts of these thermophilic proteins would be of considerable interest.

The thermophilic H^+-PPase from *Thermotoga maritima* (TVP) was the first eubacterial K^+-stimulated membrane-bound pyrophosphate to be described [7]. A partial purification procedure for TVP expressed in *E. coli* inner-membrane vesicles (IMV) has been recently reported [9]. In this work, we report the purification of a hexahistidine-tagged version of TVP after overexpression in yeast, making use of the hyperthermophilic properties of the protein. To our knowledge, this is the first H^+-PPase purified to electrophoretical homogeneity from a heterologous source. The homogeneity of the purified detergent-solubilized protein was analyzed by electron microscopy and a single particle analysis showed a dimeric organization. This study demonstrates that TVP provides new possibilities for the functional and structural analysis of H^+-PPases. Moreover, the original two-step purification procedure described herein, which takes advantage of a high-temperature solubilization strategy not previously attempted, could be used for easy and fast purification of large amounts of other thermophilic membrane proteins expressed in heterologous systems.

2. Materials and methods

All chemicals used in this work were of analytical grade.

2.1. Plasmid construction

For overexpression of TVP in yeast, the corresponding full-length *vppa* gene was amplified by PCR using plasmid pTVP [7] as a template. The primers used for this amplification – TVP1: 5′-TTT**GTCGAC**ATG*CGAGGATCACA TCATCATCATCATCAT*TAGTGGCTGCTCTTTTC-3′ and TVP2: 5′-AAA**TC-TAGA**T CAGAACAGGTGAAC-3′ – contained artificial restriction sites *Xho*I and *Xba*I (bold), respectively, to allow cloning in two different multicopy plasmids: pJR1 [7], containing the *URA-3* gene and the inducible *GAL-1* promoter, and a plasmid bearing the *LEU-2* selectable marker and the strong constitutive promoter of the *PMA1* gene. The plasmids thus obtained were named pRLTVP1 and pRLTVP2, respectively. Primer TVP1 also contained a nucleotide sequence coding for the amino acid sequence $RGSH_6$ (italicized). Thus, a hexahistidine tag was introduced after the starting methionine at the N-terminal end of TVP.

2.2. Yeast transformation and culture

Wild type *S. cerevisiae* strain W303-1A (MATa, *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) was used as a heterologous host for overexpression. Cells were transformed using the LiOAc/PEG method [10] and selected by growth on 2% agar plates in culture medium without uracil or leucine, as convenient.

For purification trials, yeast transformants bearing plasmid pRLTVP1 were grown in selective liquid media with glucose as the sole carbon source up to the stationary phase. Aliquots of 50 ml were then washed twice with water, resuspended in 1 l of pre-warmed rich galactose medium, and allowed to grow for 16 h at 30 °C with shaking (160 r.p.m.) before collection.

For large-scale purification, yeast expressing TVP from plasmid pRLTVP2 was grown in 50 ml of selective liquid medium with glucose up to the stationary phase. Several 1-l aliquots of pre-warmed rich glucose medium were then inoculated with these cultures and incubated for 18 h at 30 °C with shaking (160 r.p.m.).

2.3. Crude membrane fraction isolation

Cells were harvested by centrifugation at 3000 rpm (Beckman rotor JA14) for 5 min and washed subsequently with 1/2 and 1/10 of the original culture volume with ice-cold water. The cell pellet was resuspended in 1 ml of homogenization buffer (213 mM Tris–HCl pH 7.5, 42.5 mM EDTA pH 8.0, 42.5% [v/v] glycerol, 0.5 mM PMSF, 1 mM pepstatin A, 5 mM DTT) per 2 g fresh weight.

For purification trials, four 8-ml aliquots of cell suspension were transferred to 30-ml plastic tubes and disrupted by vigorous shaking in the presence of 12 g of glass beads (ca. 0.5 mm diameter). Cell debris and glass beads were removed by centrifugation at 4000×*g* for 10 min and the supernatant was ultracentrifuged at 100,000×*g* for 40 min. The pellet was homogenized in resuspension buffer (50 mM Tris–HCl pH 7.5, 20% [v/v] glycerol, 1.34 mM DTT, 1.34 mM $MgCl_2$, 1.5 mM PMSF, 1 mM pepstatin A). Total membrane fraction samples at a concentration of 6 mg protein/ml were used directly for solubilization or frozen in liquid nitrogen and kept at −80 °C.

For large-scale purification, a total volume of 120 ml of cell suspension and 165 g of ice-cold glass beads were taken to a Bead-Beater (Biospec Products, Bartlesville, OK) breaking chamber. The chamber was filled with GTED20 buffer (10 mM Tris–HCl pH 7.6, 1 mM EDTA pH 8.0, 20% [v/v] glycerol, 1 mM DTT) and cells disrupted by shaking 10 times for 1 min under ice with a 1-min rest between shakings. Chamber contents were then transferred to 200-ml tubes and centrifuged at 4000×*g* for 10 min to separate unbroken cells and glass beads. After ultracentrifugation of the supernatant, total membranes were homogenized in a modified resuspension buffer containing 20 mM MES at pH 6.5 instead of Tris. Immediately before solubilization, samples were supplemented with Na_2PPi 0.25 mM and KCl 1.34 mM.

2.4. Solubilization of membrane proteins

For test-solubilization of TVP from crude yeast membranes, 600 μg of total protein were solubilized with n-dodecyl-β-D-maltopyranoside (DDM) (Anatrace Inc., Maumee, OH, catalogue number D310S) at a detergent to protein ratio 3:1 (w/w). Samples were thoroughly mixed and incubated for 30 min at the desired temperature. After this time, they were taken to an air-driven ultracentrifuge (Beckman Airfuge model) in 500-μl tubes and centrifuged at 100,000×*g* for 10 min. Supernatants and pellets were collected separately and analyzed immediately or frozen in liquid nitrogen and kept at −80 °C.

For large-scale solubilization, 45-mg aliquots of total membrane protein in a volume of 30 ml were taken to 50-ml plastic tubes and heated at 75 °C for 20 min before addition of 10 ml of solubilization buffer (50 mM MES pH 6.5, 20% [v/v] glycerol, 1.35% DDM) at the same temperature. Samples were mixed by inversion and incubated at 75 °C for 2 h followed by centrifugation at 4500×*g* for 5 min. Supernatant was then transferred to fresh 50-ml tubes, placed on ice and allowed to cool for 10 min before repeating the centrifugation step. The supernatant of this last centrifugation was immediately used for Ni-NTA affinity purification of TVP.

2.5. Purification by Ni-NTA affinity binding chromatography

20-ml aliquots of solubilized fraction from the previous step were supplemented with 250 mM KCl and taken to 40 ml with resuspension buffer. These samples were incubated with 1 ml of Ni-NTA matrix (QIAGEN, catalogue number 30230) under gentle shaking at 40 °C for 1–2 h. Samples were cooled 10 min on ice before centrifugation at 3000×*g* for 3 min. Pellets were resuspended by gentle shaking in 10 ml ice-cold wash

buffer A (50 mM MES pH 6.5, 20% [v/v] glycerol, 250 mM KCl, 2.5 mM imidazole pH 6.5, 0.15% DDM, 1.5 mM PMSF, 1 mM pepstatin A, 1 mM DTT) and the centrifugation was repeated. Two more washing steps were carried out with wash buffers B (50 mM MES pH 6.5, 20% [v/v] glycerol, 50 mM KCl, 2.5 mM imidazole pH 6.5, 0.15% DDM, 1.5 mM PMSF, 1 mM pepstatin A, 1 mM DTT) and C (50 mM MES pH 6.5, 20% [v/v] glycerol, 2.5 mM imidazole pH 6.5, 0.15% DDM, 1.5 mM PMSF, 1 mM pepstatin A, 1 mM DTT). Finally, the slurry was resuspended in 2 ml of elution buffer (50 mM MES pH 6.5, 8% [v/v] glycerol, 0.5 M Imidazole pH 6.5, 0.15% DDM, 1.5 mM PMSF, 1 mM pepstatin A, 5 mM DTT) and incubated at 40 °C with gentle shaking for 5 min. A piece of glass wool was inserted in the tip of 2.5-ml syringes to use these as columns for separation of the Ni-NTA matrix from the eluted protein. Final slurry was transferred to these syringes and pure TVP was eluted by centrifugation at 1000×*g* for 5 min.

2.6. Protein quantification and PPase activity assays

Protein content was estimated by the method of Bradford [11] using gamma-globulin as a standard. PPase activity measurements were carried out as follows: a sample of crude membranes or protein in a total volume of 25 μl was taken to 160 μl with water, then, 15 μl 1 M Tris–HCl pH 8 and 15 μl 40 mM $MgCl_2$ were added. Due to the requirement of the protein for potassium for full activity, samples were supplemented with 30 μl 1 M KCl or 30 μl water as a control. These samples were incubated at 70 °C for 5 min before addition of 10 μl 10 mM Na_2PPi. The enzymatic reaction was allowed to proceed for 10 min at the same temperature. Samples were then cooled on ice for 2 min and released inorganic phosphate was quantified using the method of Baginsky et al. [12].

2.7. Protein reactivation with lipids

For reactivation of the purified TVP, fractions containing 0, 5, 10, 15, 20 or 25 μl of the imidazol-eluted protein (at 0.5 mg/ml) were mixed respectively with 25, 20, 15, 10, 5 and 0 μl of a lipid-containing buffer, obtained by mixing 170 μl of a 30-mg/ml L-α-phosphatidyl-choline (SIGMA P-5638) solution and 17 μl of 20% DDM and making up to a final volume of 500 μl with resuspension buffer. PPase activity was assayed as above. Best results were obtained with samples containing 7.5 μg of TVP and 100 μg of lipids.

2.8. Electrophoretic analysis and Western blot detection

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 10% polyacrylamide gels following the protocol of Laemmli [13]. Molecular mass standards were purchased from BioRad (Kaleidoscope pre-stained standards, catalogue number 161–0324). Gels were stained with Coomassie-blue or by reverse-Zn staining [14]. Inmunodetection (Western Blot) assays were performed by standard methods using two antibodies: PAB_{HK} [15] and a commercial antibody against the amino acid motif $RGSH_6$ (Qiagen, catalogue number 34650).

2.9. Electron microscopy and image analysis

Purified TVP protein was adsorbed onto glow-discharged carbon-coated electron microscopy grids (300 mesh) and negatively stained using 1% uranyl acetate. Electron micrographs were recorded onto a Philips CM120 electron microscope at an accelerating voltage of 120 kV and nominal magnification of 45,000, at −1 μm as defocus value. All micrographs were recorded on a 1024×1024 px Gatan ssCCD camera with a sampling window of 20 μm which results in a pixel size of 3.8 Å/pixel. A total number of 8220 projections were windowed and aligned by using X-MIPP software [16] to compute an average image. Symmetry analysis of the average image was also performed with X-MIPP by Fourier–Bessel decomposition algorithms. Self-organizing mapping (SOM), based on Kohonen neural network [17] was carried out over windowed images. Multivariate statistical analysis of dictionary vectors was used to perform the segmentation of the SOM-map and to identify the homogeneous groups of projections as described in [18]. The average image of each group was computed by using X-MIPP software after independent free-reference alignment of particles belonging to each class.

3. Results and discussion

3.1. Expression of TVP in S. cerevisiae

Purification of TVP from *E. coli* IMV had resulted only in a partially purified protein [9]. Hence the yeast *Saccharomyces cerevisiae* was used as a heterologous host in this work. Moreover, the protein had been previously expressed in this organism and shown to be resistant to yeast proteases [7]. Coomassie-blue staining of SDS-PAGE gels loaded with crude membrane fractions from yeast cells expressing a hexahistidine-tagged TVP showed galactose-induced expression of a 69-kDa polypeptide (Fig. 1). This protein could be inmunodetected with specific antibodies both against the hexahistidine tag and an epitope conserved in all known H^+-PPases (Fig. 1), which confirmed its identity as the recombinant TVP. Although the predicted molecular mass for this H^+-PPase is 73 kDa according to its amino acid sequence, several other H^+-PPases present abnormal mobilities on SDS-PAGE [19,20] and a value of 66-kDa has been reported for an untagged DDM-solubilized TVP obtained from *E. coli* IMV [9].

A specific pyrophosphatase activity around 0.15 μmol mg^{-1} min^{-1} was measured at 70 °C in crude membranes containing TVP. This PPase activity was insensitive to fluoride, dependent on the presence of Mg^{2+} and stimulated around 6-fold by K^+ ions (not shown), which is in good agreement with previous reports [7,9], indicating that the hexahistidine tag does not affect the catalytic properties of the heterologous protein.

3.2. High-temperature solubilization of TVP from crude membrane fractions

Several attempts were made to solubilize TVP from the total membrane fractions using DDM. This detergent had

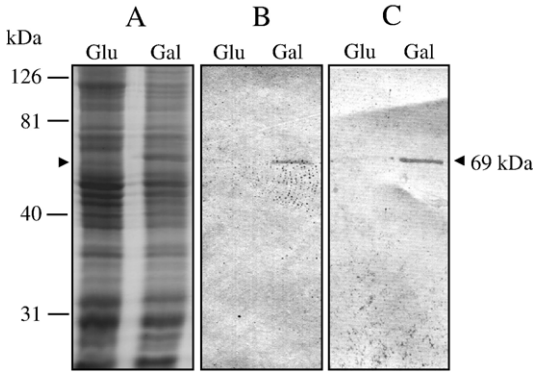

Fig. 1. Overexpression of hexahistidine-tagged TVP in yeast. TVP was overexpressed in *S. cerevisiae* under the control of yeast galactokinase (*GAL1*) promoter, which is induced by galactose and repressed by glucose. (A) Coomassie Blue staining of a SDS-PAGE gel loaded with 40 μg of total membrane protein from yeast grown with glucose (Glu) or galactose (Gal) as a carbon source. (B) Inmunodetection of TVP in the same samples using a polyclonal antibody against an epitope conserved in all H^+-PPases. (C) Inmunodetection of TVP in the samples using an antibody against the amino acid motif $RGSH_6$, introduced by PCR at the N-terminal end of the protein. Arrowheads indicate the position of TVP.

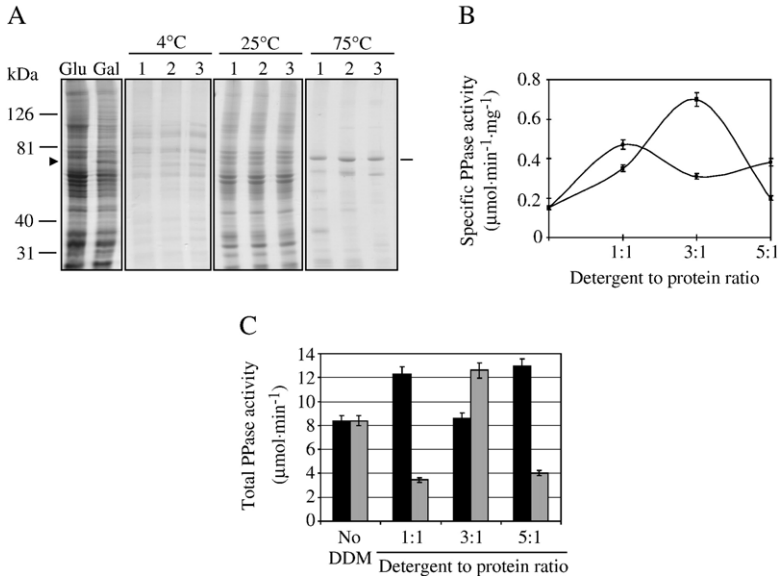

Fig. 2. Solubilization of TVP at different temperatures. Total yeast membrane fractions were solubilized with different concentrations of DDM at three different temperatures. (A) Coomassie blue staining of SDS-PAGE gels loaded with 40 μg of total membranes obtained from yeast grown in glucose (Glu) or galactose (Gal) and with 50 μl of fractions solubilized at the indicated temperatures with three different detergent to protein ratio: lanes 1, 1:1; lanes 2, 3:1; lanes 3, 5:1. (B) Specific activity of samples obtained at 25 °C (dots) and 75 °C (squares). (C) Total PPase activity recovered from 1 l of yeast culture. Black bars, solubilization temperature 25 °C; Gray bars, solubilization temperature 75 °C. No significant PPase activity could be measured for samples solubilized at 4 °C. Values are means ± S.D. of three independent experiments. Arrowheads as in Fig. 1.

been successfully used for solubilization of the protein from *E. coli* at 4 °C [9]. Solubilization of our yeast membrane preparations at this temperature resulted in a very low recovery of TVP (Fig. 2A). We rationalized that a high-temperature solubilization of the recombinant H^+-PPase could be advantageous for several reasons. First, increased temperature was assumed to increase the yield of detergent-solubilized protein. Second, high temperature was predicted to result in denaturation and aggregation of most yeast proteins, which would facilitate their separation from the thermostable TVP. Third, as the pump might be vulnerable to proteolytic attack during the purification procedure, high temperature was likely to inactivate harmful yeast proteases in the preparation.

Increasing temperatures up to the optimal value for PPase activity improved solubilization, to the extent that the protein could not be detected in the pellet obtained by ultracentrifugation of samples treated with DDM at 75 °C (not shown). Optimal solubilization temperature and detergent to protein ratio were found to be 75 °C and 3:1, respectively (Figs. 2B and C). Incubation at higher temperatures resulted in a loss of activity as previously reported [7]. Improved solubilization could be due to increased interaction between the detergent molecules and the lipids closely surrounding the thermophilic protein [21]. A heating step after solubilization at 4 °C has been previously used during purification of thermophilic proteins from heterologous membranes, including TVP [9,22] but, to our knowledge, this is the first report of a direct solubilization at high temperature ("Hot-Solve") of an integral membrane protein.

The presence of ligands, cofactors or substrates during the solubilization procedure has proven to be effective as a means for recovering the desired protein in an active state [23]. In order to check the effect of several ligands on stability of TVP during solubilization at 75 °C, different concentrations of cofactors (Mg^{2+}, K^+) and PPi were included in the samples. As TVP thermostability is strictly dependent on the presence of Mg^{2+} [7], all the trials were done in the presence of at least 1 mM $MgCl_2$. Although it was clear that the presence of the ligands stabilized the protein, no significant differences could be detected for K^+ and Mg^{2+} concentrations over 1 mM and PPi concentrations over 0.25 mM. When the three ligands were combined at the optimal concentrations during solubilization, an 8-fold purification of the protein was achieved without any loss in total PPase activity (Fig. 3).

3.3. Large-scale purification of TVP

For large-scale purification, the hexahistidine-tagged TVP was expressed under control of the strong constitutive *PMA1* promoter, in order to avoid the need to wash the cells before inoculation of the final medium. This did not seem to affect the levels of expression of the recombinant H^+-PPase, as observed by Coomassie-Blue staining of SDS-PAGE gels (not shown) and allowed the manipulation of larger volumes of culture medium. Solubilization was carried out at 75 °C in the presence of optimal concentrations of K^+, Mg^{2+} and PPi, but incubation times were increased up to 2 h to compensate for the variation in heat transfer efficiency due to sample size difference. To avoid interferences with the subsequent step of Ni-affinity purifica-

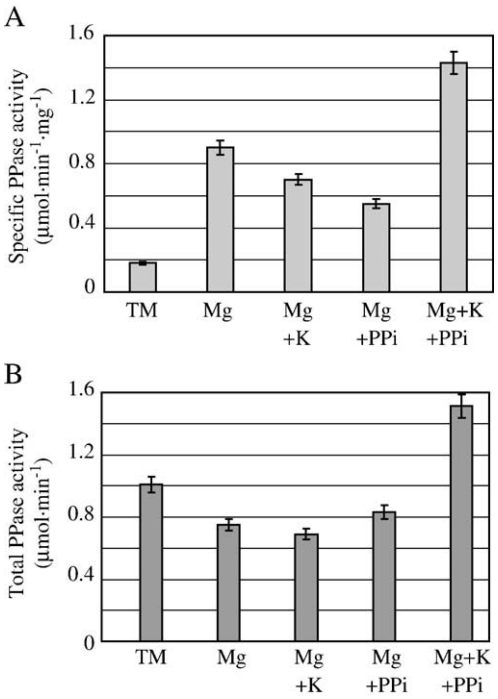

Fig. 3. Stability of TVP during solubilization in the presence of ligands. Effects of K^+, Mg^{2+} and PPi and their different combinations on protein solubilization at 75 °C. Ligand concentrations were 1.0 mM, 1.0 mM and 0.25 mM, respectively. (A) Specific activity. (B) Total activity values for 1 l of original yeast culture. In both cases, samples were incubated for 30 min before measuring the phosphate released during the enzymatic reaction. Values are means ± S.D. of three independent experiments.

tion, MES buffer was used for large-scale solubilization. The use of this buffer at pH 6.5 turned out to be very efficient. Thus, a 26-fold purification of TVP was achieved in a single step using MES as compared to the only 8-fold purification using Tris buffer (Fig. 4).

Initially, the "Hot-Solve" samples were cooled on ice and ultracentrifuged at 100,000×*g* for 1 h to remove denatured and non-solubilized proteins. As this procedure is time-consuming and most contaminating proteins present in the sample are denatured due to extensive heating, the ultracentrifugation step was replaced by two shorter centrifugations: first, solubilized samples were centrifuged at 4500×*g* for 5 min immediately after heating to eliminate most of the denatured proteins; second, supernatants were cooled on ice for ten min and another fraction of contaminants was removed by a subsequent identical centrifugation. The latter treatment took around 30 min and yielded a fairly clean solubilized fraction which could be directly used for affinity binding, by means of the hexahistidine tag introduced at the N-terminal end of the recombinant protein.

3.4. Ni-affinity binding and protein reactivation

For further purification, the hexahistidine-tagged TVP was incubated with a Ni^{2+}-charged agarose matrix. Initial experiments carried out at 4 °C or at room temperature were very inefficient as only a very small fraction of TVP could be recovered. This negative result could be explained by a compact rigid structure which would prevent the tag from being accessible to interaction with the Ni^{2+} ions [24–26], as incubations at higher temperatures resulted in a good binding to the matrix. When incubations were carried out at 40 °C, up to 1.5 mg of TVP per litre of yeast culture could be recovered by competitive elution with imidazole. The eluted fraction was subjected to SDS-PAGE electrophoresis and Zn-staining, in order to visualize contaminating proteins in the samples (Fig. 5A). This reverse-staining procedure is almost as sensitive as silver-staining with the advantage that it is not affected by the protein characteristics [14]. No contaminating proteins could be detected either by this sensitive method or by Coomassie-blue staining of gels loaded with 20 μg of purified protein (Fig. 5B).

A specific activity of 10 μmol Pi mg^{-1} min^{-1} was measured upon reactivation of the Ni-NTA eluted protein with soybean lipids, a 66-fold purification with respect to the crude membrane preparation. Another striking feature of the purified TVP is its high stability. The protein preparation was stable upon storage at room temperature in the presence of all its known ligands for about 1 week, and up to 3 weeks provided fresh DTT (1 mM final concentration) was added to the preparation every 5–6 days.

3.5. Single particle analysis of purified TVP

Electron micrographs acquired from purified TVP samples showed the presence of a very homogeneous population of

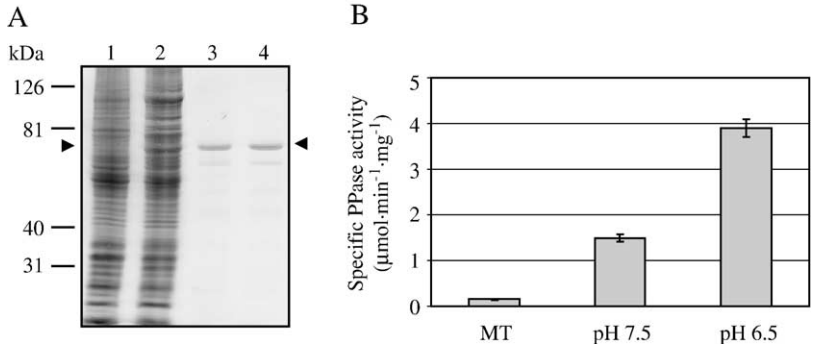

Fig. 4. Solubilization of TVP at two different pH values. (A) Coomassie-blue staining of SDS-PAGE gels loaded with total membranes (40 μg protein per lane) from cells grown on glucose (lane 1) and galactose (lane 2) and 50 μl of solubilized samples at 75 °C in Tris–HCl buffer at pH 7.5 (lane 3) or in MES-NaOH buffer at pH 6.5 (lane 4). (B) Specific activities of solubilized samples at the given pH values. Values are means ± S.D. of three independent experiments. Arrowheads as in Fig. 1.

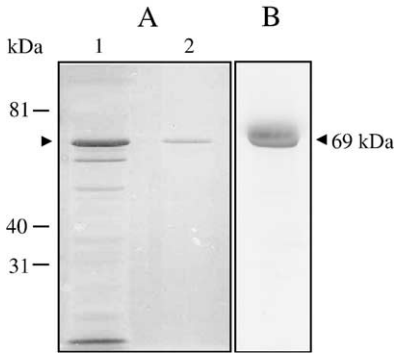

Fig. 5. Electrophoretical homogeneity of purified TVP. Different fractions of purified protein were subjected to SDS-PAGE. (A) Zinc-staining of a gel loaded with a sample of solubilized proteins (10 μg, lane 1) and 2 μg purified TVP after Ni-affinity chromatography (lane 2). (B) Coomassie-blue staining of a gel loaded with 20 μg of the purified protein.

particles having a size of around 10 nm but different shapes (Fig. 6A). The 2D-single particle analysis of these particles resulted in an almost rectangular average image (Fig. 6B), which cumulates 87% of the total symmetry in the second harmonic. The grey-level 2-fold symmetrized image shows six peripheral stain-excluding regions (noted 1 to 6 in Fig. 6C) and a less intense central one (noted 7 in Fig. 6C), all with an equivalent diameter of 2 nm. The distances between each pair of densities, measured from their periphery, are 11 nm (1–4), 9 nm (2–6 and 3–5), 10 nm (2–5 and 3–6), 2.5 nm (1–7 and 4–7) and 2 nm (2–7 and 5–7).

Self organizing mapping (SOM) analysis of the TVP images used to compute the average resulted in seven classes (Fig. 6, panels D to J) constituting percentages of 12.6 (D), 10.9 (E), 13.8 (F), 19.5 (G), 9.3 (H), 22.2 (I) and 11.7 (J). Seven stain-excluding regions are present in six of the seven classes (panels D, E, G, H, I and J), the main difference being the distance between densities. Thus, density 7 appears to be in contact with 5 and 6 (panel D), in a central position (panels E, G and J), or in contact with 4 and 5 (panel H). Regarding inter-peripheral densities, densities 1 to 6 are equidistant in most classes, except for those corresponding to panels I and J, in which densities 1 and 2 and densities 4 and 5, respectively, are in contact. Finally, the class corresponding to panel F shows only the six peripheral densities (1 to 6) and is the most asymmetrical. A first interpretation of the SOM classification could be related to a tilt of the particle on the grid. Thus, classes on panels E and G could be interpreted as horizontal views presenting staining differences. Classes corresponding to panels I and J could be understood if a tilt-axis were defined by densities 3, 7 and 6, and the TVP particles were tilted along this axis in opposite directions in both panels. Classes on D and H could be assigned to similar views where the tilt-axis is defined across the inter-density spaces. Finally, class F could correspond to the most tilted one which also results in it being the most asymmetrical. In this case, density 7 disappears under one of the six peripheral densities. A second interpretation for the SOM analysis classification could be that TVP particles are slightly deformed by collapse on the electron microscopic grid and/or by dehydration during the staining process.

Regardless of this, the distribution of the seven densities shown in Fig. 6C and the distances between them can be interpreted as the projection image of a particle composed of two domains of about 9×5 nm. At this point, it is important to recall that predictions by different online-available transmem-

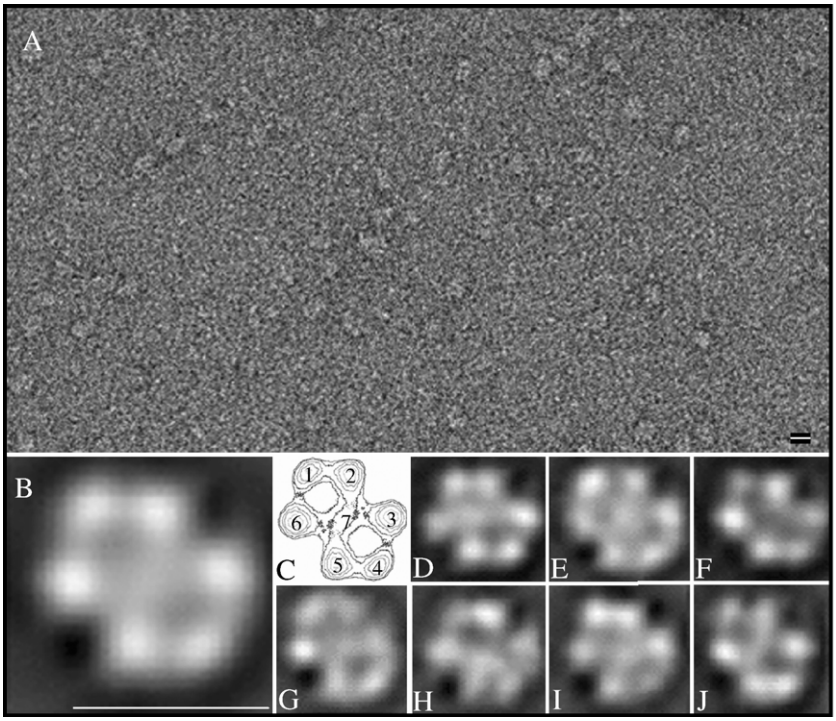

Fig. 6. Single particle analysis of purified TVP by electron microscopy. Panel A, negatively-stained transmission electron microscopy picture of a standard purified protein preparation; Panels B–J, image processing of TVP 3D-structure (see text). Bars represent ca. 10 nm.

brane helix topology predictors (TMHMM Server, v. 2.0, TOPS) indicate 16 putative transmembrane spans for the TVP protein sequence, which is in agreement with former predictions based on experimental data [27]. The size of each 9×5-nm domain in the average image obtained by electron microscopy is compatible with the surface occupied by a 16-alpha-helices membrane protein arranged in a relaxed structure. EM projection maps for monomers of different 12 alpha-helices transporters show the following sizes: 4.9×3.7 nm for melobiose permease [28], 4.8×3.8 nm for the NhaA transporter [29] and 4.8×3.2 nm for the oxalate-formate antiport protein [30]. On the other hand, a tight packing of 16 alpha-helices perpendicularly oriented would correspond to a particle of 4×4 nm with a diameter around 5 nm. Thus, 2×16 transmembrane helices may be well fitted within the two domains of the average image shown in Fig. 6C, indicating that the solubilized TVP is a dimer.

These results are in agreement with previous reports on the oligomeric state of plant vacuolar H^+-PPase as determined by indirect methods. A dimer has been repeatedly proposed to be the active oligomer for these H^+-PPases, according to studies based on gel filtration chromatography [31,32], radiation inactivation [32,33], thermo-inactivation [34], chemical cross-linking studies [35] or inactivation by high hydrostatic pressure [36]. More recently, this has also been proposed to be true for the H^+-PPase from the actinobacterium *Streptomyces coelicolor* [37]. To our knowledge, the electron micrographs presented herein are the first direct and visual evidence of the dimeric structure of a H^+-PPase.

3.6. Concluding remarks

We have developed a fast large-scale method for isolation of TVP from yeast membranes to obtain a fraction of the protein with high specific activity (ca. 10 μmol min^{-1} mg^{-1}) and purified to electrophoretical homogeneity. The stability and high yield of homogeneous TVP will be convenient for further biophysical and structural characterization, including 2-D and 3-D crystallization or mutant studies. In this context, preliminary single particle analysis studies of the purified preparation have provided the first structural images of a H^+-PPase obtained so far and have shown that TVP may well be a dimer. Finally, we propose that the two-step purification procedure presented – "Hot-Solve" followed by affinity binding chromatography – could also be extended to other recombinant thermophilic membrane proteins, opening a door to fast and easy large-scale preparation of material for biophysical, structural and mutagenesis studies.

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