

# Reconstitution of water channel function of an aquaporin overexpressed and purified from *Pichia pastoris*

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**Abstract** The aquaporin PM28A is one of the major integral proteins in spinach leaf plasma membranes. Phosphorylation/dephosphorylation of Ser274 at the C-terminus and of Ser115 in the first cytoplasmic loop has been shown to regulate the water channel activity of PM28A when expressed in *Xenopus* oocytes. To understand the mechanisms of the phosphorylation-mediated gating of the channel the structure of PM28A is required. In a first step we have used the methylotrophic yeast *Pichia pastoris* for expression of the *pm28a* gene. The expressed protein has a molecular mass of 32462 Da as determined by matrix-assisted laser desorption ionization-mass spectrometry, forms tetramers as revealed by electron microscopy and is functionally active when reconstituted in proteoliposomes. PM28A was efficiently solubilized from urea- and alkali-stripped *Pichia* membranes by octyl- $\beta$ -D-thioglucopyranoside resulting in a final yield of 25 mg of purified protein per liter of cell culture.

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**Key words:** Aquaporin; Overexpression; Electron microscopy; Mass spectrometry; Reconstitution; *Pichia pastoris*

## 1. Introduction

The plant water channel PM28A from spinach is a member of the aquaporin protein family formerly known as the major intrinsic protein (MIP) family [1–3]. The importance of these proteins is implied by their high abundance; they constitute up to 50% of total membrane protein in some membranes. Aquaporins are found in most cell types and in organisms ranging from bacteria and yeast to plants and animals [4]. The first member of this protein family for which water permeation was demonstrated was aquaporin-1 (AQP1) from human red blood cells [5]. Based on sequence comparisons, the aquaporin family can be divided into two clusters, the aquaporin (AQP) cluster and the glycerol facilitator-like proteins (GLP) cluster [6]. Members of the AQP cluster are mostly specific water channel proteins, while those of the

GLP cluster transport glycerol and/or other small solutes in addition to water [4]. All plant aquaporins map to the AQP cluster [2]. However, the sequence-based clustering into AQP and GLP proteins is unlikely to fully characterize functional distinctions since the basis for substrate specificity likely arises from changes of a few amino acids defining the constriction region of the aqueous pore [6,7].

Aquaporins are small (26–34 kDa) integral membrane proteins sharing the same overall topology with six hydrophobic membrane-spanning  $\alpha$ -helices [6]. The helices are connected by five loops and the N- and C-termini are located on the cytosolic side of the membrane. Aquaporins have two highly conserved signature motifs: the asparagine-proline-alanine (NPA) boxes, which are located in loops B and E [8]. These loops form short  $\alpha$ -helices and fold back into the membrane from opposite sides forming a seventh transmembrane domain. In the proposed ‘hourglass model’ for the aquaporin structure [8], the seventh hydrophobic domain containing the NPA boxes lines the actual pore and is likely to be involved in substrate specificity. The hourglass model has been confirmed by medium- and high-resolution three-dimensional (3D) maps of AQP1 [8–11].

The plant aquaporins constitute a large gene family. Both in *Arabidopsis* and in maize, 35 different aquaporin genes have been identified, which are divided into four subfamilies [3,12]. Two of the subfamilies seem to correspond to aquaporins with different subcellular locations. The PIPs (plasma membrane intrinsic proteins) are localized in the plasma membrane while the TIPs (tonoplast intrinsic proteins) are localized in the vacuolar membrane. The other two subfamilies are the NIPs (NOD26-like intrinsic proteins) and the SIPs (small basic intrinsic proteins) [28]. The subcellular localization of the latter two subfamilies has not been established yet. PM28A is one of the major aquaporins in spinach leaf plasma membranes where it constitutes up to 10% of all membrane proteins [1].

Aquaporins exist as tetramers in the native membrane although the monomer is the functional unit as shown for AQP1 [13]. The tetramers can be reconstituted into highly ordered two-dimensional (2D) crystals suitable for structural studies using electron and atomic force microscopy [14]. Most of the structural data acquired so far are based on aquaporins purified from their native membranes. Since plants contain about 35 highly homologous aquaporins [3], the use of a heterologous expression system avoids possible co-purification of isoforms frequently encountered when native membranes are used as starting material. This was the case for PM28A,

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which is expressed at high levels in spinach leaf plasma membrane, but could only be purified together with a PIP1 aquaporin isoform, PM28C ([15], L. Frayssé et al., unpublished results). A system for heterologous expression is also desirable as it will offer the possibility to study the structure and function of mutant forms of a protein.

The water channel activity of PM28A is regulated by phosphorylation at two sites: Ser115 in the first cytoplasmic loop and Ser274 in the C-terminal region [1,16]. Although the regulatory mechanism is not known, the constitutively high abundance of PM28A in the membrane suggests that phosphorylation mediates a direct gating of the channel. We have shown that PM28A can be phosphorylated and dephosphorylated *in vitro* by using protein kinase C and alkaline phosphatase or an endogenous partially purified calcium-dependent protein kinase ([1,16] and S. Sjövall et al., unpublished results). The heterologously overexpressed protein can also be phosphorylated by protein kinase C (M. Karlsson and S. Sjövall, unpublished results). To understand the gating mechanism, the structure of PM28A must be solved and as a first step large amounts of functional protein need to be produced and purified to homogeneity. In order to obtain sufficient amounts of PM28A for biophysical studies, we overproduced the recombinant protein in the methylotrophic yeast *Pichia pastoris*. With a recovery of about 25 mg of protein per liter of cell culture, *Pichia* gives the highest yield reported for an overexpressed aquaporin so far. Furthermore, the protein was analyzed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) and electron microscopy, and was reconstituted into proteoliposomes and shown to be functionally active.

## 2. Materials and methods

### 2.1. Plasmid construction and transformation

*P. pastoris* expression kit, including vectors and host cells, was purchased from Invitrogen. The *pm28a* cDNA (GenBank accession number L77969) was amplified with the forward primer *EcoRI*-YPM28A (5'-CGGAATTCAAAATGTCTAAGGAAGTAAGT-3') and the reverse primer *XbaI*-PM28A (5'-GCTCTAGATTGGTAGGGTTGCTTCT-3') (restriction sites underlined and yeast start codon in bold). The PCR product was cloned into the *P. pastoris* vector pPICZB and the resulting plasmid pHT-PM28A-PICZ was sequenced. The 22 amino acids added to the C-terminus of HT-PM28A are LEQKLISEEDLNSAVDHHHHHH and contain beside the 6×His tag a myc antibody epitope (bold letters). A second construct was made using the forward primer *EcoRI*-YPM28A and the reverse primer PM28A-REV (5'-GAAGATCCTTAATTGGTAGGGTTGCT-3'). The reverse primer has the original stop codon after PM28A and a *Bgl*II restriction site (underlined). The PCR product was cloned into pPICZB and the resulting plasmid pPM28A-PICZ was sequenced.

### 2.2. Expression

PM28A constructs were transformed into the wild-type *P. pastoris* strain X-33 and transformants with the highest expression according to immunostaining (TetraHis antibodies, Qiagen) were selected and grown on a large scale. Briefly, 200 ml of buffered glycerol complex medium was inoculated, and the culture was incubated at 30°C with shaking at 225 rpm overnight. The cells were pelleted by centrifugation and resuspended in 1200 ml of buffered methanol complex medium to obtain an OD<sub>600</sub> = 1.0. Cells were grown in baffled flasks at 30°C with shaking at 225 rpm. Additional (0.5% v/v) methanol was added to the culture every 24 h to maintain induction. The cells were harvested by centrifugation after 72–120 h and stored at –80°C.

### 2.3. Preparation of membranes from *P. pastoris*

Cell pellets were thawed and resuspended in breaking buffer (50

mM sodium phosphate buffer, pH 7.5, 5% glycerol). The cells were disrupted by three passages through a French pressure cell at 16000 psi and lysates were clarified by centrifugation at 3000 rpm for 30 min. Membranes were collected by centrifugation at 200000×g for 120 min at 4°C, resuspended in breaking buffer and stored at –80°C. Between 400 and 500 mg of crude membrane protein per liter of cell culture, or about 13 mg/g of cells, was routinely obtained. Peripheral membrane proteins and proteins adhering to the membranes were removed by urea/alkali treatment [15,17]. The stripped membranes were resuspended in 20 mM HEPES–NaOH, pH 7.8, 50 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol (buffer A) at a protein concentration of about 10 mg/ml and stored frozen at –80°C until further use.

### 2.4. Protein solubilization and purification

Stripped membranes containing 20 mg of protein were diluted to a protein concentration of 2 mg/ml and solubilized in 3% octyl-β-D-thioglucopyranoside (OTG) by dropwise addition from a stock solution of 10% OTG in buffer A. After 30 min at room temperature, unsolubilized material was pelleted at 160000×g for 30 min. Solubilized protein was mixed for 2 h at 4°C with 2 ml of Ni-NTA agarose slurry (Qiagen) pre-equilibrated with 20 mM HEPES–NaOH, pH 7.8, 300 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol, 0.4% OTG (buffer B) containing 10 mM imidazole. The non-bound protein fraction was removed by centrifugation and the Ni-NTA agarose was washed with buffer B+30 mM imidazole. The proteins were eluted with 1 ml of buffer B+300 mM imidazole by mixing for 1 h at 4°C. Four additional elution steps were performed. The protein concentrations were determined as described by Bearden [18].

### 2.5. Functional characterization by stopped-flow spectroscopy

Purified HT-PM28A was reconstituted into proteoliposomes by mixing with *Escherichia coli* lipids (Avanti Polar Lipids, Alabaster, AL, USA) solubilized in 7.5% OTG at a lipid-to-protein ratio (LPR) of 30, 60 and 90 in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 2 mM dithiothreitol, 0.03% NaN<sub>3</sub> and a final concentration of 2 mg lipids/ml. The mixture was dialyzed in dialyze tubings (Sigma) with a molecular cut-off of 12–17 kDa at room temperature for 5 days. Control vesicles were made in the same manner without protein. The vesicles were diluted to 0.4 mg lipids/ml, filtered through a 0.2 μm filter and subjected to an inwardly directed sorbitol gradient of 100 mM through rapid mixing in a SX.18MV stopped-flow instrument from Applied Photophysics (Leatherhead, UK). Decreased vesicle volume was measured as an increase in scattered light monitored at 90° after excitation at 500 nm and 25°C. Solubilized PM28A was reconstituted using the same protocol.

### 2.6. Mass spectral analysis of HT-PM28A

MALDI-MS analysis was performed using a Bruker REFLEX III mass spectrometer (Bruker Daltonik, Germany) as described previously [15]. The final mass and the corresponding standard deviation was calculated using the [M+H]<sup>+</sup> molecular ions of nine different spectra.

### 2.7. Transmission electron microscopy and image processing

Electron micrographs of single particles of detergent-solubilized proteins eluted from the Ni-NTA column were recorded. Image processing, single particle and multivariate statistical analysis of HT-PM28A was performed as described previously [15].

## 3. Results and discussion

To reproducibly produce crystals of AQPs in order to generate high quality structural data, it is necessary to have access to large amounts of specific AQP isoforms. The large number of aquaporin isoforms makes it a difficult task to purify a desired single isoform from native tissue. *Arabidopsis* has 35 aquaporin genes [3] with specific but overlapping expression patterns, both in space and in time. The number of isoforms is probably about the same in spinach. The purification of native PM28A led to the identification of a novel isoform, PM28C, and even though PM28A and PM28C belong

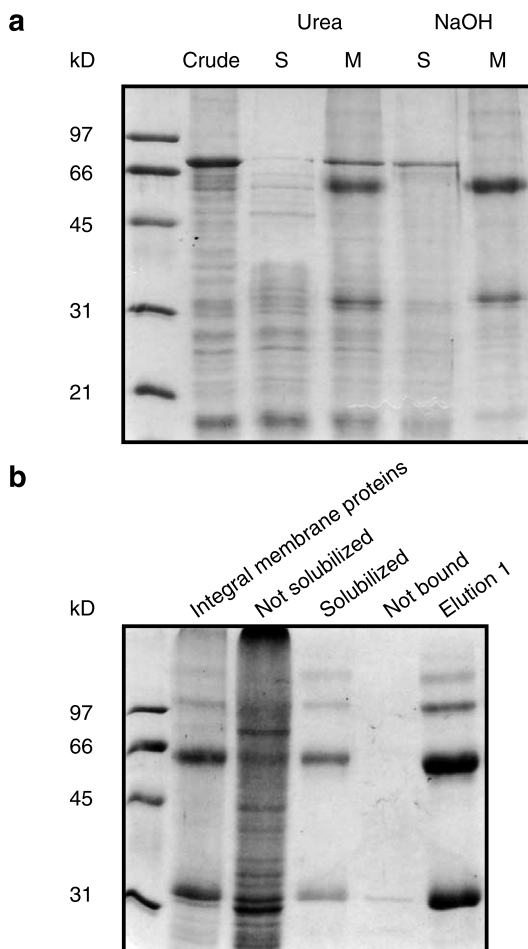


Fig. 1. a: SDS-PAGE of the crude membrane fraction, the supernatants (S) and the membrane (M) fractions from the urea and alkali washing steps. b: SDS-PAGE of the integral membrane proteins remaining after the NaOH wash (NaOH/M in a) and after solubilization with OTG and of the different purification steps of HT-PM28A by Ni-NTA affinity chromatography. Both gels were stained by Coomassie brilliant blue R-250.

to different groups of the PIP subfamily (PIP2 and PIP1, respectively) with different biochemical properties, the two proteins could not be separated [15]. This motivated us to express PM28A heterologously.

To facilitate purification and immunological detection, the gene encoding the spinach plasma membrane aquaporin PM28A was fused in-frame to DNA encoding a myc epitope and six consecutive histidines at the C-terminus generating His-tagged PM28A (HT-PM28A). The *ht-pm28a* construct was introduced at the chromosomal *AOX1* locus of the methylotrophic yeast *P. pastoris* by homologous recombination. Ten independent transformants were screened for HT-PM28A expression. Three of these clones were positive, and the clone expressing at the highest level was chosen for large-scale production. The expression levels were detected by immunostaining of cell lysates at several time points from 0 to 120 h after induction with methanol. The expression reached its maximum after 24 h and remained constant until the cells were harvested after 72–120 h (data not shown). The cells were broken using a French press and a crude membrane fraction was collected by centrifugation. Peripheral membrane

proteins and proteins adhering to the membranes were removed by urea/alkali treatment, which has been shown to enhance solubilization of integral membrane proteins [15,17,19]. The membrane fractions were analyzed by SDS-PAGE (Fig. 1a). HT-PM28A was highly enriched in the integral membrane protein fraction and only small amounts of HT-PM28A were lost in the wash supernatants according to immunoblotting experiments (data not shown). Protein solubilization was optimized for a detergent suitable for 2D crystallization of PM28A [15]. The stripped membrane fraction was efficiently solubilized in 3% OTG and the solubilized proteins consisted mainly of HT-PM28A with minor contaminants (Fig. 1b). HT-PM28A was then affinity purified using Ni-NTA agarose chromatography. Only small amounts of HT-PM28A could be detected in the fraction not bound to the Ni-NTA agarose (Fig. 1b). The tendency of HT-PM28A to form higher oligomers can also clearly be seen by SDS-PAGE (Fig. 1b). Starting with 20 mg of integral membrane proteins, about 8 mg of pure HT-PM28A could be eluted from the Ni-NTA agarose beads. This corresponds to about 25 mg of HT-PM28A per liter of cell culture or 0.7 mg per gram wet weight of cells.

The first mammalian aquaporin successfully overexpressed in a heterologous system was MIP26 from rat eye lens fiber cells [20]. The highest expression was achieved in a TOPP2 *E. coli* strain and an estimation of the yield by absorbance gave a value of 10 mg MIP26 per liter of induced cell culture. However, the yield of purified protein was not determined, and functional assays were not performed. The baculovirus-Sf9 insect cell system was used to express non-tagged AQP4 yielding 0.11 mg of purified protein per liter [21], which is insufficient for structural studies. Furthermore, the His-tagged form of AQP4 was reported to be non-functional [21]. The same system was used to express human AQP2 with a yield of 0.5 mg purified protein per liter of culture [19]. To our knowledge, the yield of functional HT-PM28A and PM28A reported in the current study (25 mg purified protein per liter)

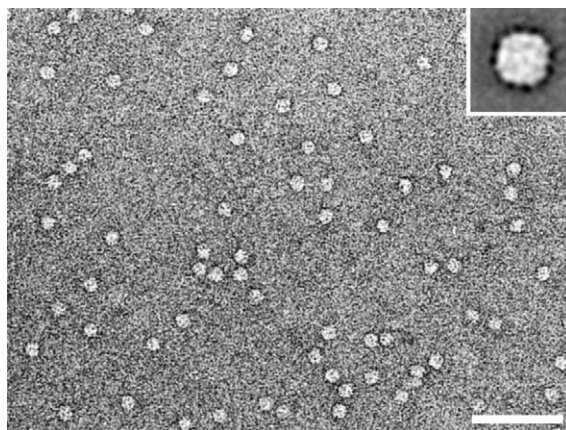


Fig. 2. Electron microscopy and image processing of detergent-solubilized HT-PM28A tetramers. The homogeneity of the preparation is reflected in the electron micrograph. After single particle and multivariate statistical analysis, an average was calculated. The average is unsymmetrized and displays the top view of HT-PM28A (inset). HT-PM28A is square-shaped, indicating its tetrameric nature. The number of averaged particles (added in the average) is  $n = 226$ . The frame size of the inset is 20 nm and the scale bar represents 65 nm.

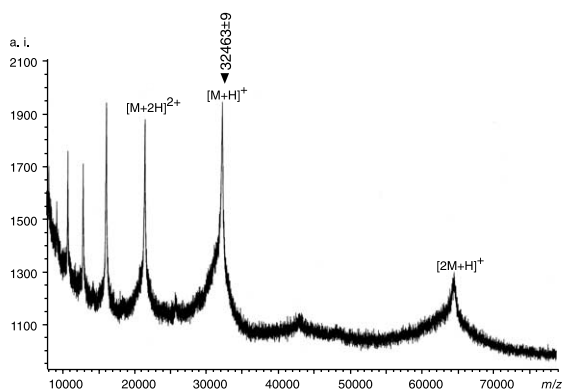


Fig. 3. Mass spectrometry of HT-PM28A reconstituted into lipid bilayers. The different molecular ions of HT-PM28A can be recognized on the MALDI spectrum. The singly charged molecular ions,  $[M+H]^+$ , the doubly charged ions,  $[M+2H]^{2+}$ , and the singly charged dimers,  $[2M+H]^+$ , of HT-PM28A are marked. A  $m/z$  of  $32463 \pm 9$  ( $n=9$ ) was measured for the singly charged HT-PM28A ion ( $[M+H]^+$ ).

is far higher than that for any of the heterologously expressed aquaporins described earlier.

Detergent-solubilized HT-PM28A proteins were negatively stained and examined by transmission electron microscopy. The homogeneity of the purified HT-PM28A after the Ni-NTA purification step can clearly be seen in Fig. 2. To enhance the signal-to-noise ratio and determine the oligomeric state of HT-PM28A, single particle and multivariate statistical analysis techniques were applied [22,23]. The inset in Fig. 2 shows the top view projection average of HT-PM28A, which displays a distinct square shape with a side length of 9.4 nm. The average was calculated from a gallery containing 703 particles that had been extracted from digitized electron micrographs by automated particle picking. The resolution of the projection average (number of particles added = 226) was determined using the following three criteria: the Fourier ring correlation function (FRC) [24], the phase residual (PHR) [25], and the spectral signal-to-noise ratio (SSNR) [26]. The average had a resolution of 1.4 nm (FRC), 2.0 nm (PHR) and 1.9 nm (SSNR). Detergent-solubilized HT-PM28As assemble as tetramers suggesting a correct folding and oligomeric assembly of the protein in *Pichia*. Compared to negatively stained native PM28A tetramers [15], the HT-PM28A tetramers appear slightly larger. The latter may arise from the extension (myc epitope and His tag) engineered to the C-terminus of HT-PM28A and from *Pichia* lipids tightly bound to the protein.

Mass spectrometry was performed on purified HT-PM28A after being reconstituted into lipid bilayers. The MALDI spectrum in Fig. 3 displays only peaks corresponding to the differently charged states of the HT-PM28A protein. Neither contamination nor degradation of the protein was observed indicating that the expressed protein is stable and not degraded by proteases during purification. MALDI analysis yielded a mass of  $32462 \pm 9$  Da ( $n=9$ ) for the entire HT-PM28A protein. Subtraction of the mass of the additional 22 amino acids added at the C-terminus in the construct ( $6 \times$  His tag and the myc antibody epitope; see Section 2), results in a mass of 29836 Da for the PM28A protein. This fits with the mass of 29838 Da measured for the native PM28A isolated from spinach leaf plasma membranes [15].

Attempts to phosphorylate and dephosphorylate HT-PM28A with protein kinase C and alkaline phosphatase, respectively, prior to mass determination were undertaken. However, the resolution was not high enough to resolve such minute mass differences. In vitro phosphorylation assays with a crude membrane fraction from *Pichia* mixed with  $[^{32}\text{P}]\text{ATP}$  showed that endogenous kinases in *Pichia* are able to phosphorylate HT-PM28A. This implies that the recombinant HT-PM28A may be phosphorylated to some extent when expressed in *Pichia*.

Functional measurements of osmotic water permeability using crude and stripped membrane fractions from wild-type and HT-PM28A-expressing *Pichia* failed. A possible explanation why stopped-flow measurements were not successful might be that these membrane fractions did not form vesicles spontaneously [27]. To assay the water channel activity of HT-PM28A, the protein was reconstituted into proteoliposomes. Vesicles were obtained reproducibly when purified HT-PM28A was reconstituted in *E. coli* lipids. Functional measurements of osmotic water permeability were carried out by stopped-flow light spectroscopy. The HT-PM28A proteoliposomes were subjected to a 100 mM sorbitol inwardly directed osmotic gradient, and the time course of the scattered light was recorded at 25°C. The water channel activity was

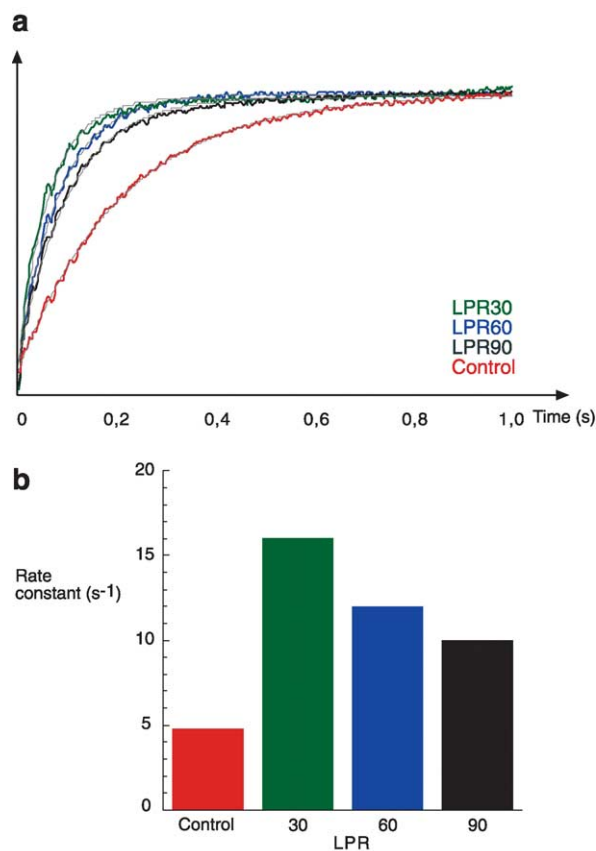


Fig. 4. a: Proteoliposomes reconstituted at different LPRs (30, 60, 90) and control liposomes were abruptly exposed to an osmotic gradient of 100 mM sorbitol. Light scattering due to water efflux causing vesicle shrinkage was monitored in a stopped-flow apparatus. The light scattering traces of > 10 runs at each LPR of HT-PM28A proteoliposomes and control liposomes were averaged and fitted to single exponential equations. b: The rate constants of the fitted single exponential equations of proteoliposomes at LPR 30, 60 and 90 and of control liposomes.

compared to controls, i.e. liposomes without protein. The traces from >10 individual runs were averaged and the curves were fitted to single exponential equations. Fig. 4a shows the averaged traces for HT-PM28A proteoliposomes at LPRs of 30, 60 and 90, and the control. The initial rate constants of the fitted equations of the proteoliposomes were LPR 30: 16.02 s<sup>-1</sup>, LPR 60: 11.94 s<sup>-1</sup>, LPR 90: 9.94 s<sup>-1</sup> and for the control 4.77 s<sup>-1</sup> as displayed in Fig. 4b.

In order to further establish the functionality of recombinant PM28A, a clone expressing PM28A without the His tag and the myc antibody epitope was constructed. This clone expressed PM28A at similar high levels. Stripped and solubilized membrane proteins from this *Pichia* clone consist of >95% PM28A, as judged by SDS-PAGE (data not shown). The protein from the solubilized stripped membrane fraction could therefore be reconstituted for activity comparisons without further purification. PM28A was reconstituted into *E. coli* lipids at LPR 30, 60, and 90 and water permeability measurements were carried out by stopped-flow light spectroscopy in the same manner as for HT-PM28A. PM28A proteoliposomes had the same characteristics as the HT-PM28A proteoliposomes, indicating that the His tag and the myc antibody epitope of HT-PM28A do not affect the activity and that the protein was correctly folded despite the extra amino acids (data not shown).

Here we demonstrate that a plant membrane protein can be expressed at high levels in *P. pastoris*. This recombinant protein, HT-PM28A, was stable, could be solubilized in OTG and reconstituted in *E. coli* lipids. Its functional activity was demonstrated by light scattering experiments. The expression and purification method described here is a solid basis for functional studies of mutated PM28A and for structural analyses using 2D and 3D crystals.

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