Structural Characterization of Two Aquaporins Isolated from Native Spinach Leaf Plasma Membranes*

Two members of the aquaporin family, PM28A and a new one, PM28C, were isolated and shown to be the major constituents of spinach leaf plasma membranes. These two isoforms were identified and characterized by matrix-assisted laser desorption ionization-mass spectrometry. Edman degradation yielded the amino acid sequence of two domains belonging to the new isoform. PM28B, a previously described isoform, was not found in our preparations. Scanning transmission electron microscopy mass analysis revealed both PM28 isoforms to be tetrameric. Two types of particles, a larger and a smaller one, were found by transmission electron microscopy of negatively stained solubilized proteins and by atomic force microscopy of PM28 two-dimensional crystals. The ratio of larger to smaller particles observed by transmission electron microscopy and single particle analysis correlated with the ratio of PM28A to PM28C determined by matrix-assisted laser desorption ionization-mass spectrometry. The absence of PM28B and the ratio of PM28A to PM28C indicate that these plasma membrane intrinsic proteins are differentially expressed in spinach leaves. These findings suggest that differential expression of the various aquaporin isoforms may regulate the water flux across the plasma membrane, in addition to the known mechanism of regulation by phosphorylation.

Water is the universal solvent and most important molecule for life. Immense water volumes pass across the membranes of all living cells, especially across the plasma membranes of plants (1). Simple diffusion of water through the lipid bilayer has an activation energy of >10 kcal/mol and cannot explain the rapid water flow through human red cell membranes found to exhibit an activation energy of <5 kcal/mol. This led to the hypothesis that water pores must exist (2). The discovery (3) to exhibit an activation energy of 5 kcal/mol. This led to the hypothesis that water pores must exist (2). The discovery (3), the rapid water flow through human red cell membranes found (4) of the first known water channel, aquaporin-1 (AQP1), from human erythrocytes and the demonstration of water transport in Xenopus oocytes expressing its complementary RNA (5) confirmed this hypothesis.

Since then, a large number of channel-forming integral proteins homologous to AQP1 have been found in all forms of life (6). This membrane protein family was initially named the MIP family after its first sequenced member, the major intrinsic protein (MIP) of bovine lens fiber cells (7). Multiple sequence alignment and phylogenetic analysis of 164 members of the MIP family, now frequently referred to as aquaporin superfamily, revealed 16 subfamilies that form two distinct clusters, the aquaporin (AQP) cluster and the glycerol facilitator-like cluster (8). The AQPs are highly specific for water, whereas the glycerol facilitators allow the passage of small, nonionic molecules such as glycerol and urea (9). In addition, ovine MIP tetratramers have been found to form a groove and tongue contact via their extracellular surfaces, lending support to a dual function of the protein, as a water channel and as a cell to cell adhesion molecule in the eye lens (10).

Most members of the aquaporin super family have similar molecular masses, ranging from 25 to 31 kDa. Based on their sequence homology all members are predicted to comprise six hydrophobic, membrane-spanning α-helices connected by five loops of variable length and to have cytosolic N and C termini (7, 11). Highly conserved regions are located on the loops B and E, which contain the NPA amino acid motifs (12). Site-directed mutagenesis experiments led to the hypothesis that loops B and E fold back into the membrane and that the NPA boxes must be involved in the selectivity filter of the channel (13). Other highly conserved residues are found in the helices, revealing the transmembrane helix-helix packing motif GXXG (14), as well as conserved charged buried residues that were proposed to form ion pairs (15).

Velocity sedimentation, glutaraldehyde cross-linking, and gel filtration (16), as well as transmission and scanning transmission electron microscopy (STEM) (17, 18), have shown that AQPs and glycerol facilitators are tetrameric proteins. When reconstituted into lipid bilayers, these proteins often form highly ordered two-dimensional (2D) crystals suitable for cryoelectron and atomic force microscopy (AFM). Consequently, a

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1 The abbreviations used are: AQP, aquaporin; [M + H]+, protonated molecular mass; 2D, two-dimensional; AFM, atomic force microscopy; FRC, Fourier ring correlation function; MALDI, matrix-assisted laser desorption ionization; MS, 2-(N-morpholino)ethanesulfonic acid; MIP, major intrinsic protein; MS, mass spectrometry; OTG, octyl-β-D-thio- glucopyranoside; PIP, plasma membrane intrinsic protein; pHR, phase residual; HPLC, high-performance liquid chromatography; SSNR, spectral signal to noise ratio; STEM, scanning transmission electron microscopy; TIP, tonoplast intrinsic protein; TOF, time of flight; Bicine, N,N-bis(2-hydroxyethyl)glycine; TBST, Tris-buffered saline with Tween 20.

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wealth of structural information is now available for several aquaporins from different organisms, ranging from single particle projections to the atomic model elucidated at 0.38-nm resolution (19) and subnanometer topographical data recorded by AFM (see Ref. 18).

Cell to cell water transport is required for many physiological processes in plants, including the transcellular movement of water in the transpiration stream, the circulation of water between the xylem and the phloem, the stomatal or organ movement, and cell enlargement. Aquaporins have been found in the plasma membrane (plasma membrane intrinsic proteins (PIPs)) and in the vacuolar membrane (tonoplasm intrinsic proteins (TIPs)) of plants, being expressed in organ-, tissue-, and cell type-specific manners. At the cellular level PIPs act in water uptake and release, whereas TIPs are thought to mediate cellular turgor maintaining the structural integrity of the cell. Plant PIPs are further divided into two subfamilies named PIP1 and PIP2 (20). In addition to several single amino acid residue substitutions, PIP1s are characterized by a long N terminus and a shorter C terminus and PIP2s by a short N terminus and a longer C terminus (21). In contrast to PIP2, PIP1 and PIP2 (20). In addition to several single amino acid residue substitutions, PIP1s are characterized by a long N terminus and a shorter C terminus and PIP2s by a short N terminus and a longer C terminus (21). In contrast to PIP2, PIP1s are characterized by a long N terminus and a shorter C terminus and PIP2s by a short N terminus and a longer C terminus (21).

Here, we present the structure of PM28A and a new putative aquaporin isoform, PM28C, whose sequence is distinct from that of PM28B but is also a member of the PIP1 subfamily. A fast and efficient purification protocol likely to be applicable to other plant membranes has been developed. The oligomeric state of PM28A and PM28C particles is defined by STEM mass analysis, and their shape, dimensions, and distribution are defined by electron and atomic force microscopy. In addition, the exact mass and stochiometry of the mixture containing the two isoforms was determined by matrix-assisted laser desorption/ionization mass-spectrometry (MALDI-MS). The absence of PM28B in our preparations and the ratio of PM28A to PM28C indicate that these PIPs are differentially expressed in spinach leaf plasma membranes.

EXPERIMENTAL PROCEDURES

**Chemicals**—*Escherichia coli* lipids were purchased from Avanti Polar Lipids (Alabaster, AL), octyl-β-D-thiogalactopyranoside (OTG) was from Anatrace (Maumee, OH), and endoproteinase Lys-C (Achromobacter protease) was from Wako Chemicals GmbH (Neuss, Germany).

**Copurification of Two Plant Aquaporin Isoforms**—Preparation of plasma membranes from spinach (*Spinacia oleracea*) leaves was performed as described in Ref. 27. Proteins adhering to the plasma membranes were solubilized by a urea/alkali treatment (22, 29). Briefly, membrane proteins (protein concentration, 14–18 mg/ml) were homogenized in 100 ml of 5 mM Tris-HCl (pH 9.5), 5 mM EDTA, 5 mM EGTA, 4 % urea, 0.01% NaN3, at 4 °C and was centrifuged (40 min, 4 °C, 100,000 × g in a Kontron TPF 45.94 rotor). The pellets were taken up in 100 ml of 20 mM NaOH (pH ~12), homogenized at 4 °C, and centrifuged again. Finally, the pellet obtained was homogenized with 100 ml of 5 mM Tris-HCl (pH 8), 2 mM EDTA, 2 mM EGTA, 100 mM NaCl, 0.01% NaN3 (storage buffer) at 4 °C and was pelleted by centrifugation. These urea/alkali-stripped plasma membranes were resuspended at a protein concentration of 2–3 mg/ml in storage buffer and stored frozen at −80 °C until further use. After thawing, stripped plasma membranes were incubated with 3% OTG (stock solution, 10% OTG in 10 mM Hepes-NaOH (pH 7.6), 0.01% NaN3) for 30 min on a shaker at room temperature. After centrifugation (40 min, 4 °C, 117,000 × g in a Beckman TLA 100 rotor), the supernatant was loaded onto a MiniS column (Amersham Pharmacia Biotech) connected to a Smart™ chromatography station (Amersham Pharmacia Biotech). The column was equilibrated with 20 mM Bicine-NaOH (pH 8.75), 0.4% OTG, 0.008% NaN3. Proteins were desorbed from the MiniS column using a 0 to 250 mM NaCl gradient in 20 mM Bicine-NaOH (pH 8.75), 0.4% OTG, 0.008% NaN3, over 80 min at a flow rate of 100 μl/min. Fractions containing PM28 were identified by SDS gel electrophoresis (30), pooled, and concentrated to 2 mg/ml in Centricon-100 cartridges (Amicon, Beverly, MA). Protein concentrations were determined using the BCA assay (Pierce).

**SDS Polyacrylamide Gel Electrophoresis and Immunoblotting**—Solubilized or reconstituted PM28 was incubated in sample buffer containing 1.5% SDS and 10% β-mercaptoethanol for 10 min at room temperature, run on 13% polyacrylamide gels (30), and silver-stained. Gels were electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were blocked for 30 min in 2% milk powder dissolved in 10 mM Tris-HCl (pH 8), 150 mM NaCl, 0.05% Tween 20, 0.02% NaN3 (TBST), and was subsequently incubated for 60 min with the primary antibody (serum-diluted 1:2000 in TBST) directed against the sequence ALSFRSRTNPPT in the C-terminal region of PM28A (see Table I). Before incubation with the anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (Sigma; diluted 1:2000 in TBST) containing 2% milk powder and 3 times for 10 min in TBST. Blots were washed three times in TBST for 10 min, once with 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl2, and were then developed with Western Blue™ (Promega) stabilized substrate for alkaline phosphatase. All washing and incubation steps of the blot were at room temperature.

**Two-dimensional Crystallization**—Purified protein (2 mg/ml) was mixed with *E. coli* lipids solubilized in OTG (mixed micelles stock solution, 40 mg/ml *E. coli* lipids in 20 mM Mes-NaOH (pH 6), 5% OTG, 0.01% NaN3) in a lipid to protein ratio of 1 (w/w). The final protein concentration was adjusted to 1.33 mg/ml, and the final OTG content was adjusted to 1.93%. The reconstitution mixture (60 μl) was preincubated at room temperature for 30 min and dialyzed against 1.5 liters of 10 mM Mes-NaOH (pH 6), 100 mM NaCl, 100 mM MgCl2, 2 mM dithiothreitol, 0.01% NaN3 for 24 h at room temperature, 24 h at 37 °C, and another 24 h at room temperature.

**Enzymatic Digestion and Reverse-phase Chromatography**—Solubilized PM28 (0.3 mg/ml in 20 mM Bicine-NaOH (pH 8.75), 150 mM NaCl, 0.4% OTG, 0.008% NaN3) was digested with the endoproteinase Lys-C at an enzyme to substrate ratio of 1:20 (w/w) at 37 °C for 1 h. The reaction was stopped by adding trifluoroacetic acid to a final concentration of 0.4%.

**N-terminal Peptide Sequencing**—Automated Edman degradations were performed on an Applied Biosystems 473A protein sequencer according to the manufacturer’s recommendations.

**Sequence Analysis**—Alignment between PM28 sequences were performed using the program ClustalX v1.64b (31).

**Mass Spectral Analysis**—MALDI-TOF analysis was performed on a...
PM28 Plant Aquaporins

**Fig. 1.** A, silver-stained SDS polyacrylamide gel of spinach leaf plasma membranes before (lane 1) and after urea/alcohol/NaCl treatment (lane 2). The arrow indicates the position of the 28-kDa polypeptide band containing the MIPs of spinach leaf plasma membranes. B, cation exchange chromatography of stripped plasma membranes after solubilization in 3% OTG. PM28 was eluted using a 0–250 mM NaCl gradient in 20 mM Bicine-NaOH (pH 8.75), 0.4% OTG, 0.008% NaN₃. **Continuous line,** elution profile monitored at an UV absorbance of 280 nm. **Broken line,** NaCl gradient. C, silver-stained SDS polyacrylamide gel of the collected fractions. D, Western blot probed with an antibody directed against the C terminus of PM28A.

Brucker REFLEX III mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). PM28 reconstituted into lipid bilayers was solubilized for less than 20 s in 80% formic acid at room temperature (final protein concentration, 4 mg/ml). After solubilization of the membranes, 2 μl were immediately mixed with 1 μl of matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid (Aldrich) in 80% acetonitrile, 0.1% trifluoroacetic acid) and placed on the sample plate to dry. Calibration of the instrument in the high molecular mass range was done with bovine serum albumin (Sigma) by using the molecular masses of the singly, doubly, and triply charged forms of bovine serum albumin. Calibration and mass measurements of the PM28 proteins were carried out in the linear mode. The [M+H]⁺ values of PM28 isoforms shown in Fig. 7B, the ratio between the two peaks, and the corresponding standard deviations were calculated from 13 different spectra. Mass spectra for the endopeptidase Lys-C fragments of PM28 were acquired by mixing 1 μl of the fractions collected by reverse-phase HPLC with 1 μl of matrix solution and spotting the mixture onto the target plate. For mass measurement in the low molecular range the instrument was operated in the reflectron mode and calibrated using the monoisotopic masses of doubly, and triply charged forms of bovine serum albumin. Calibration in the high molecular mass range was done with bovine serum albumin (Sigma) by using the molecular masses of the singly, doubly, and triply charged forms of bovine serum albumin. Calibration and mass measurements of the PM28 proteins were carried out in the linear mode. The [M+H]⁺ values of PM28 isoforms shown in Fig. 7B, the ratio between the two peaks, and the corresponding standard deviations were calculated from 13 different spectra. Mass spectra for the endopeptidase Lys-C fragments of PM28 were acquired by mixing 1 μl of the fractions collected by reverse-phase HPLC with 1 μl of matrix solution and spotting the mixture onto the target plate. For mass measurement in the low molecular range the instrument was operated in the reflectron mode and calibrated using the monoisotopic masses of the adrenocorticotropic hormone (fragment 18–39; Fluka), substance P (Fluka), and angiotensin (Fluka).

**Scanning Transmission Electron Microscopy Mass Measurement—** PM28 isoforms solubilized in OTG were adsorbed for 1 min to glow discharged thin carbon films supported by a thick fenestrated carbon grid (directly after cation-exchange chromatography). The gold-plated membranes after solubilization in 3% OTG. PM28 was eluted using a 0–250 mM NaCl gradient in 20 mM Bicine-NaOH (pH 8.75), 0.4% OTG, 0.008% NaN₃. **Continuous line,** elution profile monitored at an UV absorbance of 280 nm. **Broken line,** NaCl gradient. C, silver-stained SDS polyacrylamide gel of the collected fractions. D, Western blot probed with an antibody directed against the C terminus of PM28A.

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**Scanning Transmission Electron Microscopy Mass Measurement—** PM28 isoforms solubilized in OTG were adsorbed for 1 min to glow discharged thin carbon films supported by a thick fenestrated carbon layer (directly after cation-exchange chromatography). The gold-plated copper grids were then washed on 8 drops of quartz double-distilled water and were freeze-dried at ~80 °C overnight in the microscope. For mass analysis, annullar dark-field images were recorded in a STEM (VG-HB5) at 80 kV and doses of 325 ± 35 electrons/nm². Digital acquisition of the images and microscope parameters, system calibration, and mass analysis were carried out as described previously (32). The total experimental error was calculated as the standard error of the mean, plus 5% of the measured particle mass to account for the absolute calibration uncertainty.

**Transmission Electron Microscopy and Image Processing—** Detergent-solubilized particles eluted from the cation-exchange column were directly adsorbed for 1 min to parlodion carbon-coated copper grids rendered hydrophilic by glow discharge at low pressure in air. Grids were washed with 4 drops of double-distilled water and stained with 2 drops of 0.75% uranyl formate. Images were recorded on Eastman Kodak Co. SO-163 sheet film with a Hitachi H-7000 electron microscope operated at 100 kV. Electron micrographs of single particles adsorbed to the carbon film were digitized using a Leafscan-45 scanner (Leaf Systems, Inc., Westborough, MA).

All image processing steps described below were performed using the Semper image processing system (33) (Synoptics Ltd., Cambridge, United Kingdom). For single-particle analysis PM28 complexes were extracted, aligned, and classified. Briefly, a reference was established by selecting a well preserved particle and symmetrizing it 20-fold rotationally (34, 35). Cross-correlation functions of this reference with images of digitized micrographs containing adsorbed particles of PM28 revealed correlation peaks at the particle positions, irrespective of their angular orientation. Using this particle picking method a gallery of 4096 particles was created. These were submitted to a multivariate statistical analysis (36) without alignment and were classified into clusters of particles with similar features. To this end, a program package kindly provided by J. P. Bretaudière (37) was used. The various cluster averages revealed square and round shaped particles at different angular orientations. These averages were taken as references for subsequent angular and translational alignment of the extracted 4096 particles. Aligned particles were classified again, and cluster averages were calculated. The resolution of the calculated averages was estimated according to the Fourier ring correlation function (FRC) (38), the phase residual (PHR) (39), and the spectral signal to noise ratio (SSNR) (40).

**Atomic Force Microscopy—** The stock solution of crystals (1.33 mg/ml protein) was diluted 20-fold in 10 mM Tris-HCl (pH 8.8), 150 mM KCl, 25 mM MgCl₂ or 10 mM Tris-HCl (pH 8.8), 50 mM MgCl₂, depending on the experiment. A 30-μl drop of this solution was deposited on freshly cleaved muscovite mica (Mica New York Corp., New York, NY) prepared as described previously (41). After 15 to 30 min, the sample was gently washed with the appropriate buffer to remove membranes that were not firmly attached to the substrate. Images were acquired with a commercial AFM (Nanoscope III; Digital Instruments, Santa Barbara, CA) equipped with a 120-μm scanner (j-scanner) and a liquid cell. The liquid cell was operated without an O-ring seal. Oxide-sharpened Si₃N₄ tips were used, and atomic force microscopy images were recorded using the semi-contact mode.
cantilevers from Olympus (Tokyo, Japan) with a length of 100 μm and a force constant of k = 0.1 newton/m were used. AFM images were recorded in the contact mode with forces between 50 and 150 piconewton applied to the tip. The imaging buffer was optimized according to Ref. 42.

RESULTS

The polypeptide pattern of spinach plasma membranes exhibits a dominant protein band of 28-kDa molecular mass (Fig. 1A, lane 1, arrow). In the first purification step, proteins adhering to the membranes were removed by urea/alkali treatment (Fig. 1A, lane 2). This stripping yielded a pure plasma membrane preparation highly enriched in membrane proteins. New bands appeared above the 42.7- and the 66.2-kDa markers, indicating aggregation of the very dominant polypeptide otherwise running at 28 kDa (Fig. 1A, lane 2). Moreover, the lipid bilayer not accessible to the detergent before the urea/alkali treatment could now be solubilized by OTG and other detergents (e.g. dodecyl-β-maltoside and octyl-β-D-glucopyranoside; data not shown). When a membrane preparation solubilized in 3% OTG was applied to a cation-exchange column, a prominent peak was observed in the elution profile at a concentration of about 55 mM NaCl (Fig. 1B). Fractions showing UV absorbance at 280 nm were collected and subjected to SDS polyacrylamide gel electrophoresis and Western blotting using an antibody directed against the C terminus of PM28A. The polypeptide bands in the silver-stained SDS gel (Fig. 1C) corresponded to the bands from the Western blot (Fig. 1D) and could, therefore, be assigned to PM28A. As seen in Table I, the peptide used to raise the PM28A-specific antiserum is depicted in bold. The tendency of PM28A to form higher oligomers can also clearly be seen in the Western blot. If the cation exchange column was not overloaded with OTG-solubilized plasma membranes, SDS polyacrylamide gel electrophoresis of the flow-through showed complete depletion of the band at 28 kDa, indicating full binding of the PM28A sequence to the column matrix (data not shown).

Table I

| PM28A | A I K A L G S F R S N P T N |
|-------|-----------------------------|
| PM28B | I P F K S R S               |
| PM28C | I P F K S K                 |

To check the purity of isolated PM28A further, the protein pool eluted from the cation exchanger was cleaved with the endoproteinase Lys-C, and the digest was separated by reverse-phase HPLC. The m/z values measured by MALDI-TOF for the fractions of the various elution peaks are indicated in Fig. 2. Fractions that could be identified as fragments coming from PM28A (Fig. 2, underlined m/z values) are listed in Table II. Fractions that could not be identified based on the measured mass were subjected to amino acid sequencing (Fig. 2, peaks marked with an asterisk). The fragments at m/z 2406.3 and m/z 4165.5 yielded blank spectra in the mass spectrometer. Elution was monitored at a UV absorbance of 214 nm. Peaks marked with an asterisk were subjected to amino acid sequencing. The fragments at m/z 2406.3 and m/z 4165.5 could be successfully sequenced by Edman degradation and yielded the amino acid sequences GFQGPYQVGGGSNYVHHGWYTK and QIN-WNWNNHFWVWVFPFIGA, respectively.

The peptide used to raise the PM28A-specific antiserum is depicted in bold. The masses of the peptides measured by MALDI-TOF in the corresponding fractions. Masses that could be assigned to the known PM28A isform are underlined (for more details see Table II). Peaks labeled with B indicate components originating from the buffer-containing detergent (20 mM Bicine-NaOH (pH 8.75), 150 mM NaCl, 0.4% OTG, 0.008% NaN₃). The elution peaks labeled with N yielded blank spectra in the mass spectrometer. Elution was monitored at a UV absorbance of 214 nm. Peaks marked with an asterisk were subjected to amino acid sequencing. The fragments at m/z 2406.3 and m/z 4165.5 could be successfully sequenced by Edman degradation and yielded the amino acid sequences GFQGPYQVGGGSNYVHHGWYTK and QIN-WNWNNHFWVWVFPFIGA, respectively.

FIG. 2. C18 reverse-phase chromatography of endoproteinase Lys-C-digested PM28. The numbers above the peaks indicate the masses of the peptides measured by MALDI-TOF in the corresponding fractions. Masses that could be assigned to the known PM28A isform are underlined (for more details see Table II). Peaks labeled with B indicate components originating from the buffer-containing detergent (20 mM Bicine-NaOH (pH 8.75), 150 mM NaCl, 0.4% OTG, 0.008% NaN₃). The elution peaks labeled with N yielded blank spectra in the mass spectrometer. Elution was monitored at a UV absorbance of 214 nm. Peaks marked with an asterisk were subjected to amino acid sequencing. The fragments at m/z 2406.3 and m/z 4165.5 could be successfully sequenced by Edman degradation and yielded the amino acid sequences GFQGPYQVGGGSNYVHHGWYTK and QIN-WNWNNHFWVWVFPFIGA, respectively.

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Mass measurement by STEM was used to assess the homogeneity of the purified PM28 isoforms and determine their aggregation state. A typical low dose dark-field image recorded from freeze-dried, unstained PM28 is shown in Fig. 4A. The mass analysis of 1169 particles yielded a single peak at 157 ± 56 kDa after correction for beam-induced mass-loss. This is compatible with a tetrmeric protein embedded in a detergent micelle of about 40 kDa. Trimeric or pentameric complexes could be excluded, because the total experimental error of the mean amounted to ± 8 kDa (for details see “Experimental Procedures”).

Detergent-solubilized PM28 particles were negatively stained and examined by transmission electron microscopy. Fig. 5A shows the homogeneity of the purified PM28 isoforms after the cation exchange chromatography step. However, two particle types with subtle differences could be distinguished upon close inspection of the electron micrographs, one type being larger and rather circular (Fig. 5B) and the other smaller and almost square (Fig. 5C). Single-particle and multivariate statistical analysis was applied to improve the signal to noise ratio. Top view projection averages for the two particle types

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2 L. Fraysse and P. Kjellbom, unpublished results.
were calculated from a gallery of 4096 particles extracted from digitized electron micrographs by automated picking. Both populations were clearly tetramers as already predicted by STEM mass measurement, consequently the averages were 4-fold symmetrized. The projection of the larger and rather circular PM28 isoform (Fig. 5B, (i) unsymmetrized and (ii) 4-fold symmetrized particles) calculated from 444 motifs of 4096 had a side length of 8.8 nm and showed less density in the center of the tetramer than at the periphery. The smaller and almost square PM28 isoform was less abundant (Fig. 5C, (i) unsymmetrized and (ii) 4-fold symmetrized particles). The projection was calculated from 255 motifs of 4096, had a side length of 8.0 nm, and had a homogeneous density distribution over the whole particle. The resolution of the projection averages was determined using the following three criteria: (i) the FRC (38), (ii) the PHR (39), and (iii) the SSNR (40). The average of the larger and rather circular particle type (Fig. 5B) had a resolution of 1.8 (FRC), 2.4 (PHR), and 1.6 nm (SSNR), whereas the smaller and almost square one (Fig. 5C) had a resolution of 1.8 (FRC), 2.5 (PHR), and 2.6 nm (SSNR).

2D crystals formed when PM28 was reconstituted into lipid bilayers. Two different crystal types were found; one with a p42,2 symmetry and lattice vectors of a = b = 9.76 ± 0.06 nm (Fig. 6A) and the other with a hexagonal lattice of closely packed tetramers and unit cell dimensions of a = b = 8.58 ± 0.18 nm (Fig. 6B). Imaging in the AFM, which has a much higher signal to noise ratio than the transmission electron microscope, indicated segregation of the two isoforms into two different crystal types and confirmed the structural features of the isoforms revealed by single particle analysis. The tetramers in the p42,2 crystal could be correlated to the smaller and almost square PM28 isoform (Fig. 5C), whereas the particles in the hexagonal lattice corresponded to the larger and rather circular one (Fig. 5B). Crystals containing a mixture of both particle types were not observed. The surface topograph of the p42,2 crystal (Fig. 6A and inset) shows some similarity to the topograph of aquaporin-Z from E. coli (43). Also the unit cell dimensions of the crystal and the side length of the particles

| Position in sequence | [M + H]+ in daltons from sequence | [M + H]+ in daltons from MALDI-TOF | Sequence of identified fragment |
|----------------------|----------------------------------|-----------------------------------|--------------------------------|
| 4–16                 | 1449.7                           | 1449.6                            | EVSSEAQAHQKG                   |
| 17–33                | 1919.9                           | 1920.3                            | DYVDPFAPFDDLSEKL                |
| 143–161              | 1913.9                           | 1913.5                            | GPYQQFGGGANVSYLQNYK             |
| 238–270              | 3733.0                           | 3733.8                            | VWDDQIPSYFIQGAAYQYVLRAAAIK      |
| 271–281              | 1163.6                           | 1163.4                            | ALGSFNPSTN                     |

**FIG. 3.** Topology of PM28A and comparison with the fragments of the new isoform PM28C. The amino acid residues shown in reverse typeface indicate sequences that could be identified by MALDI-MS and amino acid sequencing. The scissors indicate the cleavage sites for endoproteinase Lys-C. The fragments at m/z 2406.3 and m/z 4165.5 (see Fig. 2) form part of loop C and parts of loop E and helix 6, respectively, of the new aquaporin isoform isolated from spinach leaf plasma membranes. The 6 transmembrane α-helices are represented according to Ref. 19.

**TABLE II**

Endoproteinase LysC fragments of PM28A identified by mass spectrometry

| Position in sequence | [M + H]+ in daltons from sequence | [M + H]+ in daltons from MALDI-TOF | Sequence of identified fragment |
|----------------------|----------------------------------|-----------------------------------|--------------------------------|
| 4–16                 | 1449.7                           | 1449.6                            | EVSSEAQAHQKG                   |
| 17–33                | 1919.9                           | 1920.3                            | DYVDPFAPFDDLSEKL                |
| 143–161              | 1913.9                           | 1913.5                            | GPYQQFGGGANVSYLQNYK             |
| 238–270              | 3733.0                           | 3733.8                            | VWDDQIPSYFIQGAAYQYVLRAAAIK      |
| 271–281              | 1163.6                           | 1163.4                            | ALGSFNPSTN                     |

**FIG. 4.** Mass analysis of the PM28 isoforms by STEM. A, elastic dark-field STEM image of freeze-dried, OTG solubilized PM28 particles. B, mass histogram of 1169 particles selected from images recorded at an average dose of 325 ± 35 electrons/nm². After the data have been corrected for beam-induced mass-loss, a mass of 157 ± 56 kDa was obtained. The scale bar in A represents 100 nm.
of particles added 5.1 (A) and 1.0 nm (B sizes of the insets, 7.5 (A) and 10.0 nm (B). Vertical brightness ranges, represents 65 nm. The frame size in galleries A, AFM topographs, the particles displayed a less pronounced symmetry and lattice vectors of a tetramer. This central indentation correlates with the low density found in the larger and rather circular projections (gallery B). After single particle and multivariate statistical analysis of PM28 isoforms, averages were calculated. The unsymmetrized (−) and the 4-fold symmetrized (+) averages are shown in B (number of particles added = 444; side length, 8.8 nm) and C (number of particles added = 255; side length, 8.0 nm). The scale bar in A represents 65 nm. The frame size in galleries B and C is 16 nm.

forming it are almost identical to those found for aquaporin-Z (44). Particles forming the hexagonal crystal were less compact and were characterized by a deep indentation in the center of the tetramer. This central indentation correlates with the low density found in the larger and rather circular projection average obtained by single-particle analysis (Fig. 5B). Also in AFM topographs, the particles displayed a less pronounced square shape, although four corners could frequently be seen at higher magnification (Fig. 6B and inset). The tetramers seem to be flexible, allowing the formation of a hexagonal lattice, which has never been observed before for aquaporin crystals.

Mass analysis by MALDI-TOF was performed on the reconstituted membranes to measure the exact mass of the mature proteins and to exclude the existence of additional isoforms, e.g. PM28B. Fig. 7A shows the MALDI spectrum of PM28 isoforms. Two singly charged molecular ions, [M+H]+, were recorded, one at m/z 29839 ± 11 (n = 13) and the other at m/z 30683 ± 7 (Fig. 7B, n = 13). In addition to the [M+H]+, the doubly charged ion, [M+2H]2+, and the singly charged dimer, [2M+H]+, of PM28A and the new isoform were observed (Fig. 7A). No additional peaks, which would indicate the presence of other proteins, were found. Hence, the purification strategy described in the present report yields PM28A and PM28C preparations of high purity.

DISCUSSION

The urea/alkali stripping of spinach leaf plasma membranes proved to be a crucial step in our purification protocol. Not only was the amount of nonmembrane protein contaminants considerably reduced, but the lipid bilayers were also made accessible to the solubilizing detergent, enabling highly efficient solubilization. Because the plasma membranes of various plants and their tissues can be isolated by two-phase partitioning (27), this purification step should also facilitate the isolation of both their aquaporins and other membrane proteins.

The aquaporin PM28A (1) from spinach leaf plasma membranes was identified by Western blot and MALDI-MS analysis of endoproteinase Lys-C-digested PM28A peptide fragments. The latter analysis also led to the discovery of a new isoform, PM28C, that copurifies with PM28A. The amino acid sequences of two domains of this isoform were acquired by Edman degradation. Comparison revealed a high homology between corresponding domains of PM28A and PM28C, especially at the beginning of helix 6 (see Fig. 3). The deduced amino acid sequence of the new isoform, PM28C, has been shown to be distinct from that of PM28B. However, both PM28B and PM28C belong to the Pip1 subfamily, whereas PM28A belongs to the Pip2 subfamily of plasma membrane AQPs.2 These PM28 proteins have very similar masses and high calculated pI values (pI ~10) and should therefore copurify together. PM28B, which was originally identified by screening of a spinach leaf cDNA library (1) and was never isolated as a protein, could not be detected in our preparations. This indicates that either the protein is present in very low amounts or is not located in the plasma membrane of spinach leaves. Another explanation for this observation could also be that PM28B is only expressed in plasma membranes under certain, unknown stress conditions.

Both isoforms were shown to be tetramers by STEM mass analysis. The measured mass of 157 ± 56 kDa is in good agreement with the values determined for other detergent-solubilized MIP homologs by STEM (17, 28, 45). The total experimental error of ± 8 kDa is explained by counting statistics of the scattered electrons and calibration errors. Thus, the ~3.5-kDa mass difference for the tetramer of the two isoforms calculated from the MALDI-TOF measurement was not resolved (Fig. 4B). However, electron microscopy of negatively stained OTG-solubilized PM28 isoforms revealed two different populations of particles. The most abundant form consisted of the smaller and rather circular tetramers, and the less abundant form consisted of the larger and almost square projections with side lengths of 8.8 and 8.0 nm, respectively. Because particles from digitized electron micrographs were picked automatically (see “Experimental Procedures”), the number of
particles used to calculate the averages in Fig. 5, B and C was a direct indication for their distribution on the grid. The ratio of the smaller and almost square to the larger and rather circular particles was 0.6 as estimated from single-particle classification. This estimate is in excellent agreement with the ratio of the two peaks observed by MALDI-MS (Fig. 7B) of 0.6 ± 0.1 (n = 13; m/z 29839 ± 11 and the other at m/z 30683 ± 7), indicate the masses of the mature isoforms.

Crystal formation was observed after reconstitution of PM28 into lipid bilayers. Structural analysis by AFM clearly showed that the two PM28 isoforms segregate into two different crystal types; amazingly, mixed crystals were never observed. In addition, the structural features indicated by single-particle analysis were confirmed. The smaller and almost square tetramers built highly ordered 2D arrays with a p42_12, whereas the larger and rather circular particles formed less well ordered hexagonal arrays. The quality of the two crystals is reflected by the standard deviations of the unit cell dimensions (p42_12, a = b = 9.76 ± 0.06 nm; hexagonal lattice, a = b = 8.58 ± 0.18 nm), which was three times higher for the hexagonal crystal. The overall shape of the particles forming the hexagonal arrays was more round than square. Although corners were often observed on AFM topographs recorded at high magnification (Fig. 6B and inset), there was considerable flexibility in the shape of these particles. This structural inhomogeneity, on the one hand, enabled the formation of a hexagonal crystal, which is unusual for aquaporins, and on the other hand, hindered the growth of highly ordered crystals.

MALDI-MS and more recently electrospray ionization-MS have been applied to assess the masses of full-length membrane proteins with high accuracy (46–48). We have used a similar approach to test the purity of our reconstituted PM28 into 2D crystals. MALDI-MS analysis of entire aquaporins reconstituted into lipid bilayers yielded very sharp, symmetrical peaks and small standard deviations when the m/z values were averaged over several spectra. Single isoforms differing by only 844 Da could easily be resolved in the spectra. Broad, asymmetrical peaks in the MALDI spectra arise from formylated states of the protein. Thus, it was crucial to keep the incubation time of the sample in formic acid as short as possible. A very interesting perspective for the future is to demonstrate whether the resolution achieved by this method is sufficient to detect differently phosphorylated states of whole aquaporins or other membrane proteins.

PIP1 and PIP2 aquaporins were isolated from spinach leaf plasma membranes and were shown to be differentially expressed. These findings suggest that differential expression may provide a means to regulate the water flux across the plasma membrane, in addition to the known mechanism of regulation by phosphorylation of the PIP2 aquaporins (24).
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