Active Plasma Membrane P-type H⁺-ATPase Reconstituted into Nanodiscs Is a Monomer

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Background: The plasma membrane H⁺-ATPase generates electrochemical gradients in plants and fungi. The minimal subunit organization required for activity is not known.

Results: We developed a protocol for reconstitution of active H⁺-ATPase in nanodiscs.

Conclusion: The minimal functional unit of the H⁺-ATPase is a monomer.

Significance: The plasma membrane H⁺-ATPase functions like well characterized cation pumping P-type ATPases.

Plasma membrane H⁺-ATPases form a subfamily of P-type ATPases responsible for pumping protons out of cells and are essential for establishing and maintaining the crucial transmembrane proton gradient in plants and fungi. Here, we report the reconstitution of the Arabidopsis thaliana plasma membrane H⁺-ATPase isofrom 2 into soluble nanoscale lipid bilayers, also termed nanodiscs. Based on native gel analysis and cross-linking studies, the pump inserts into nanodiscs as a functional monomer. Insertion of the H⁺-ATPase into nanodiscs has the potential to enable structural and functional characterization using techniques normally applicable only for soluble proteins.

The plasma membrane (PM)3 H⁺-ATPase is a prominent member of the P-type ATPases, a large superfamily of proteins pumping ions and lipids across cellular membranes. This family of proteins forms a phosphorylated intermediate during the catalytic cycle, hence P-type, and is divided into five subfamilies, P1 to P5, each having different substrate specificities (1, 2).

The PM H⁺-ATPase belongs to the P3 subfamily only found in plant and fungi and is responsible for proton extrusion out of the cell. The resulting proton gradient is used by proton-coupled transporters and secondary active transport of nutrients and metabolites across the PM. In addition to the fundamental role in nutrient uptake, the plant PM H⁺-ATPase is involved in a number of processes during plant growth and development, such as stomatal movement and cell elongation. Also, responses to both biotic and abiotic stresses often require the activation of the PM H⁺-ATPase (3–5).

A large body of information on the structure, function, and regulation of PM H⁺-ATPases from the P3 subfamily has been compiled during the last decade. Cryo-electron microscopic images of the Neurospora crassa H⁺-ATPase (6) and the recently solved crystal structure of the Arabidopsis thaliana H⁺-ATPase isoform 2 (AHA2) (7) revealed the presence of 10 transmembrane helices and three large cytoplasmic domains, including the phosphorylation and ATP-binding sites. The overall structure of AHA2 resembles the one known for the well studied sarco/endoplasmic reticulum Ca²⁺-ATPase pumps, which belongs to the P₂ subfamily (1).

Oligomerization is a common feature of members of the P-type ATPase family of pumps. Thus, representative members of the P-ATPase family, such as the sarcoplasmic reticulum Ca²⁺ pump (8) and the Na⁺/K⁺ pump (9), self-associate. The monomer of the sarcoplasmic reticulum Ca²⁺-ATPase and the α-β protomers of the Na⁺/K⁺ - and H⁺/K⁺-ATPases are capable of performing all the steps of the reaction cycle (8, 10, 11).

The human PM Ca²⁺-ATPase is a P2B-ATPase that resembles PM H⁺-ATPases by having an extended C-terminal regulatory domain. PM Ca²⁺-ATPase isolated from human erythrocytes undergoes reversible, enzyme concentration-dependent oligomerization (12, 13). This oligomerization process involves the C-terminal calmodulin-binding domain of the pump (13–15) and likely results in an activated high affinity state of the pump (16).

An early study concluded that the functional unit of the fungal Neurospora PM H⁺-ATPase reconstituted with excess lipid...
in liposomes might be a monomer (17). Subsequent structural studies have revealed the presence of PM H⁺-ATPase dimers and hexamer complexes, but their functional roles remain to be elucidated (18, 19). A distinct characteristic of P-type PM H⁺-ATPases is the presence of a C-terminal regulatory domain (R-domain) (7), and activation of pump activity occurs by phosphorylation-dependent binding of 14–3-3 regulatory proteins to this domain (20, 21). A three-dimensional reconstruction of purified PM H⁺-ATPase/14-3-3 complex suggested a hexameric arrangement (19) in line with the reported structure from x-ray crystallography on two-dimensional crystals (22, 23). Whether the activation of the pump correlates with its oligomerization status is not known. Likewise, it remains uncertain whether the monomeric state is active like it is in other P-type ATPases.

Reconstitution of membrane-embedded proteins in soluble nanoscale lipid bilayers, also termed nanodiscs, is a novel technique in the study of membrane proteins (24). Assembled from membrane scaffold proteins (MSPs), a nanodisc consists of two MSPs encircling a planar lipid bilayer in a double-belt configuration. The amphiphilic helical structure of the MSPs shields the hydrophobic edge of the lipid bilayer and stabilizes discrete disc sizes determined by the length of the MSPs, resulting in diameters ranging from 10 to 17 nm (25, 26). Advantages in using this system include water solubility, monodispersity, flexible lipid composition, access to both sides of the bilayer simultaneously, and controlled stoichiometry of disc to target protein. Despite still being a relative novel approach for membrane protein study, several examples of membrane proteins reconstructed into nanodiscs exist, e.g. G-protein-coupled receptors (27–31) and cytochrome P450s (32–35). Nanodisc-reconstituted membrane proteins can essentially be handled as soluble proteins in aqueous solution. This facilitates the application of analytical techniques that are normally difficult to use in the study of membrane proteins such as surface plasmon resonance (SPR), nuclear magnetic resonance spectroscopy, as well as electron paramagnetic resonance spectroscopy.

Here, we demonstrate the reconstitution of the A. thaliana PM P-type proton ATPase isoform 2 into the nanodisc. Using native gel analysis, cross-linking, and transmission electron microscopy, we show that the nanodisc-embedded AHA2 is an active monomer. Reconstitution of the PM H⁺-ATPase into nanodiscs enables further functional and structural analysis of this family of pumps in complex with regulatory proteins. This is demonstrated by the ability of soluble nanodiscs with reconstituted PM H⁺-ATPase to be selectively immobilized for SPR analysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG) were used as received from Avanti Polar Lipids Inc. (Birmingham, AL). Bio-Beads SM-2 absorbent was purchased from Bio-Rad. Detergent n-dodecyl β-D-maltoside (DDM) was obtained from Glycon Biochemicals (Luckenwalde, Germany). Unless indicated otherwise, all other chemicals and reagents were obtained from Sigma.

Expression and Purification of Plasma Membrane H⁺-ATPase Isoform 2—A plasmid based on the multicopy vector YEp351 (36) was used for expression in yeast, containing the coding sequence of a 73-amino acid C-terminal truncated A. thaliana PM H⁺-ATPase isoform 2 (aha2A73) (plasmid pMPI280) (37). The coding sequence is under the control of the PM1 promoter and in fusion with a C-terminal Met-Arg-Gly-Ser-His₆ (MRGSH₆) tag. For expression, the Saccharomyces cerevisiae strain RS-72 (MATα ade1-100 his4-519 leu2-3,112) (38) was transformed and cultured essentially as described previously (39). In RS-72, the endogenous yeast PM H⁺-ATPase PM1 gene is placed under the control of a genomic galactose-dependent promoter. This strain grows in media containing galactose, whereas growth in glucose-based medium requires the complementation of the yeast PM H⁺-ATPase by the constitutively expressed A. thaliana PM H⁺-ATPase. The cells were grown and harvested by centrifugation, and membranes were isolated as described previously (7). All subsequent manipulations were performed at 4 °C, and all buffers contained 0.3 mM phenylmethylsulfonyl fluoride and 3 μg/ml pepstatin A. Membranes were solubilized using DDM at a 3:1 detergent/protein (w/v) ratio in solubilization buffer (50 mM Mes-KOH, pH 6.5, 20% (v/v) glycerol, 1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol) containing 1.2 mM ATP with gentle agitation for 30 min. Insoluble material was removed by centrifugation for 60 min at 100,000 × g. The supernatant containing solubilized A. thaliana H⁺-ATPase was diluted 1:1 (v/v) with washing buffer WB50 (50 mM Mes-KOH, pH 6.5, 20% (v/v) glycerol, 0.15% (w/v) DDM, 500 mM KCl, 10 mM imidazole) and incubated for 16 h with nickel-nitritolactriatic acid resin (1 ml of resin, 30 mg of membrane protein) pre-equilibrated in the same buffer. To minimize unspecific binding, the nickel-nitritolactriatic acid resin was washed with 10 volumes of washing buffer WB250 (as WB500 with 250 mM KCl) followed by 10 volumes of washing buffer WB50 (as WB500 with 50 mM KCl). Bound proteins were eluted with 2 volumes of elution buffer (50 mM Mes-KOH, pH 6.5, 50 mM KCl, 300 mM imidazole, 20% (v/v) glycerol, 0.5 mM dithiothreitol, and 0.075% (w/v) DDM). Centrifugal concentrators (Vivaspin 20, GE Healthcare) with a 30-kDa molecular mass cutoff were used for buffer exchange of the eluted proteins to solubilization buffer containing 0.075% (w/v) DDM in two washing steps. Purified H⁺-ATPase was finally concentrated to 5–10 g/liter, frozen in liquid nitrogen, and stored at −80 °C.

Expression and Purification of Membrane Scaffold Proteins—His₆-tagged membrane scaffold protein MSP1D1 (25) was expressed in Escherichia coli and purified as described previously (24). The His₆ tag on MSP1D1 was removed by treatment with His₆-cleaved tobacco etch virus protease, overnight at room temperature. After cleavage, the sample was loaded on a nickel-Sepharose column (GE Healthcare), and the cleaved membrane scaffolding protein MSP1D1(−) devoid of the polyhistidine tag was collected in the flow-through, whereas tobacco etch virus protease and any uncleaved protein were retained onto the column.

**Vesicle Reconstitution and Proton Pumping Assay**—Purified PM H⁺-ATPase was reconstituted into preformed asolectin vesicles as described previously (40), using 12 μg of purified protein per 200 μl of Mes-KOH buffer, pH 6.5, containing 50
mm octyl glucoside and 10.6 mm asolecin. Proton pumping was determined using 9-amino-6-chloro-2-methoxycacidine (ACMA) quenching assay (41). Briefly, proteoliposomes (20 μl) in 2 ml of buffer (20 mM MOPS-KOH, pH 7.0, 40 mM K₂SO₄, 25 mM KNO₃, 1 μM ACMA, 60 mM valinomycin, 2 mM ATP) were analyzed spectrophotometrically (excitation at 412 nm; emission at 480 nm; bandpass 2 nm, integration time 0.1 s) using a Fluoro-max-4 spectrofluorometer (Horiba, Edison, NJ). Proton pumping resulting in ACMA fluorescence quenching was initiated by the addition of MgSO₄ to a final concentration of 2 mM and when indicated was dissipated by the addition of 10 μM carbonyl cyanide m-chlorophenylhydrazone.

Reconstitution and Purification of PM H⁺-ATPase in Nanodiscs—Reconstitution of PM H⁺-ATPase into nanodiscs was based on the method described previously (24). Lipids in chloroform were mixed in glass tubes to achieve the indicated lipid composition. A lipid mixture of POPC/POPG (3:2, molar ratio) was applied in the reconstitution experiments. Excess chloroform was blown off with nitrogen, and lipids were solubilized at a concentration of 50 mM in MEK buffer (Mes-KOH, 1 mM EDTA, 100 mM KCl, pH 6.5) containing 200 mM octyl glucoside and incubated with purified MSP1D1 or MSP1D1(−) with gentle agitation overnight at 4°C. Purified aha2Δ73 was added to give a final lipid/MSP/H⁺-ATPase molar ratio of 64:1:0.125 and incubated for 1 h at 4°C. Detergent was removed by incubation for 4 h at 4°C with 1 g/ml SM-2 Adsorbent Bio-Beads. Reconstituted nanodiscs were purified and analyzed by size exclusion chromatography (SEC) (Superdex 200 10/300 GL, GE Healthcare) with a flow rate of 0.4 ml/min using MEK buffer as the mobile phase. The column was calibrated according to the manufacturer’s instructions using the following known standards: blue dextran (2 MDA); ferritin (400 kDa; 12.2 nm); aldolase (158 kDa; 9.62 nm); conalbumin (75 kDa); ovalbumin (43 kDa; 6.1 nm); carbonic anhydrase (29 kDa); ribonuclease A (13.7 kDa; 3.28 nm), and aprotinin (6.5 kDa) (GE Healthcare). Values for the Stokes diameter of selected standards were plotted at their respective elution volume. Stokes diameter of the reconstituted nanodiscs was estimated by a linear fit of the partition coefficient Kₛₐₚ versus the Stokes diameter of the standard proteins. The reconstituted nanodiscs was estimated by a linear fit of the partition coefficient Kₛₐₚ versus the Stokes diameter of the standard proteins.

Surface Plasmon Resonance Analysis—Binding analysis of ND-AHA2 was detected by SPR using a Biacore X100 instrument at 25°C. The penta-His antibody (Qiagen) was covalently immobilized to the CM5 sensor chips (GE Healthcare, Biacore) by amino coupling. The penta-His antibody was buffer-exchanged with Bio-Spin 30 columns (Bio-Rad) to 10 mM sodium acetate, pH 5.0, buffer before injection. The surface was activated by 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 0.05 M N-hydroxysuccinimide for 7 min at a flow rate of 10 μl/min. The penta-His antibody (0.1 mg/ml) was then injected for 7 min at a flow rate of 5 μl/min, and the surface was deactivated by 1 M ethanolamine-HCl for 7 min at a flow rate of 10 μl/min. The immobilization of penta-His antibody was done in a standard running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% surfactant P-20, 3 mM EDTA). The penta-His antibody was immobilized only in flow cell 2; flow cell 1 was left unmodified as a reference surface. To capture aha2Δ73 in nanodiscs on the sensor chip, the running buffer was changed to 50 mM MOPS, pH 7.5, 150 mM KCl, 1 mM DTT, 0.5 mM EDTA. Samples were diluted to different concentrations in running buffer. Each sample was injected for 380 s at a flow rate of 5 μl/min, and dissociation was observed for 600 s. The sur-
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face was regenerated by a 30-s injection of 10 mm glycine, pH 2.0, and washed with buffer for 300 s before the next injection. In each case, the bulk effect on the sensorgram was corrected by subtraction of the reference surface sensorgram from the sensorgram of the active surface functionalized with penta-His antibody and subtraction of a blank run.

Other Procedures—Proteins were analyzed by SDS-PAGE using the system of Fling and Gregerson (47). Western blotting was performed using monoclonal anti-penta-His antibodies (Qiagen), polyclonal antibodies raised against the N-terminal region of AHA2 (AHA2N term; number 762) (48), polyclonal antibodies raised against the preserved catalytic domains of AHA2 (AHA2cat) (49), polyclonal antibodies raised against a 19-amino acid peptide near the C terminus of human ApoA-1 (50), polyclonal antibody and subtraction of a blank run.

Expression in Yeast

Purification and analysis of plant PM H^+-ATPase aha2Δ73. A purified aha2Δ73 was subjected to SDS-PAGE (lane 1) and Western blot analysis using anti-His (lane 2) and anti-AHA2N term (lane 3) antibodies. B, BN-PAGE analysis of purified aha2Δ73. Lane M, NativeMark protein standards (Invitrogen); lane 1, DDM-solubilized aha2Δ73. The three major bands, X1, X2, and X3, are labeled by arrowheads. C, chemical cross-linking of aha2Δ73. 2-Fold dilutions of aha2Δ73 (starting amount ~5 µg) were incubated with DMS as indicated and subjected to SDS-PAGE analysis. The cross-linking product is specified by an arrowhead. D, proton pumping of vesicle-reconstituted aha2Δ73 monitored by the proton-dependent fluorophore ACMA. Pumping is initiated by the addition of MgSO_4.

RESULTS

Plant PM H^+-ATPase Purifies as a Dimer upon Heterologous Expression in Yeast—The S. cerevisiae strain RS-72 has previously proved to be suitable for the high level expression and purification of A. thaliana PM H^+-ATPase AHA2 (50). Here, we used this strain for expression and purification of a C-terminally truncated version of AHA2 (aha2Δ73), which lacks 73 amino acid residues at the C terminus. This deletion removes a regulatory autoinhibitory domain of AHA2 and renders it in a constitutively activated state (39). Furthermore, aha2Δ73 can be expressed in higher yields as compared with AHA2 and is less prone to degradation (data not shown). Under our conditions, a typical purification by Ni^{2+} affinity batch-binding resulted in ~1–2 mg of protein from 1 liter of cell culture. The purified PM H^+-ATPase had the expected molecular mass of 97 kDa and was homogeneous as assessed by Coomassie Brilliant Blue-stained SDS-PAGE (Fig. 1A). The protein identity was unequivocally confirmed by Western blot analysis using the anti-His and anti-AHA2N term antibodies (Fig. 1A). ATPase analysis of the purified aha2Δ73 in detergent-containing buffer in the presence of asolectin or POPC/POPG (3:2 molar ratio) revealed an average ATPase activity of 10–20 µmol of P_i/min/mg of protein.

To evaluate the oligomeric state of purified PM H^+-ATPase, aha2Δ73 was subjected to BN-PAGE analysis. The protein migrated predominantly as a single band with a size of ~200 kDa (Fig. 1B, X1) in the range of dimeric aha2Δ73, with weaker bands visible around ~450 kDa (Fig. 1B, X2) and ~600 kDa (Fig. 1B, X3). To confirm the oligomeric states of aha2Δ73, cross-linking was performed using the amine-specific cross-linker DMS (Fig. 1C). Following incubation with DMS, PM H^+-ATPase migrated as two bands in SDS-PAGE; the PM H^+-ATPase, aha2Δ73, with weaker bands visible around ~450 kDa (Fig. 1B, X2) and ~600 kDa (Fig. 1B, X3). To confirm the oligomeric states of aha2Δ73, cross-linking was performed using the amine-specific cross-linker DMS (Fig. 1C). Following incubation with DMS, PM H^+-ATPase migrated as two bands in SDS-PAGE; the PM H^+-ATPase monomer at ~97 kDa and a band migrating above 170 kDa (indicated by an arrow in Fig. 1C) near the molecular mass correspond to dimeric aha2Δ73 of about 194 kDa. Both bands were recognized in Western blots using an anti-AHA2cat antibody (data not shown). 2-Fold dilutions of the starting amount of PM H^+-ATPase resulted in similar dimeric/monomer ratios, excluding concentration effects as a possible explanation for the presence of dimers. Furthermore, some protein retained at the stacking gel, suggesting the formation of higher oligomers or aggregates too large to enter the gel pores. These data suggest that the majority of DDM-solubilized PM H^+-ATPase is present as oligomers.
Upon reconstitution into asolectin vesicles, aha2Δ73 exhibited ATP-driven proton pumping activity as revealed by ATP-dependent fluorescence quenching of ACMA, a dye that accumulates inside the vesicles upon protonation (Fig. 1D). These results demonstrate successful purification of functionally active PM H⁺-ATPase.

Reconstitution of PM H⁺-ATPase into Nanodiscs—Purified aha2Δ73 was used for reconstitution into nanodiscs employing the MSP construct MSP1D1 or MSP1D1(−) devoid of the His₇ tag. An MSP/aha2Δ73 ratio was chosen to give a 4-fold excess of nanodiscs to PM H⁺-ATPase, facilitating the reconstitution of monomeric PM H⁺-ATPase. Assembly of stable PM H⁺-ATPase-nanodisc complexes from MSP, lipids, and detergent-solubilized PM H⁺-ATPase was verified by size exclusion chromatography. For nanodisc preparation with PM H⁺-ATPase, the chromatogram displayed a major elution peak with a shoulder on the leading edge of the peak (Fig. 2A). By contrast, nanodisc preparation without PM H⁺-ATPase showed a large and symmetrical elution peak. SDS-PAGE analysis revealed that the PM H⁺-ATPase co-elutes with MSP in the shoulder region, although the major peak corresponds to MSP alone representing empty nanodiscs (Fig. 2B). Analysis of the ATPase activity across the elution fractions revealed that the peak of enzymatic activity coincided with ND-AHA2 complexes in the shoulder region (Fig. 2C). Taken together, these results confirm successful reconstitution of functional PM H⁺-ATPase into nanodiscs.

Separation of Empty and Full Nanodisc Complexes—From initial reconstitution attempts, it was evident that a single size exclusion chromatography step was insufficient to separate ND-AHA2 complexes from empty nanodiscs, i.e. devoid of PM H⁺-ATPase. To allow for purification of ND-AHA2 complexes, purified His₇-tagged aha2Δ73 was reconstituted into nanodiscs using the MSP construct MSP1D1(−) devoid of the polyhistidine tag and subsequently subjected to Ni²⁺ affinity chromatography (Fig. 3A). Analysis of the proteins eluted from the Ni²⁺ matrix by SDS-PAGE and Western blotting demonstrated that under this condition, ND-AHA2 bound to the Ni²⁺ matrix, although the flow-through contained mainly the empty nanodiscs, with residual aha2Δ73 as observed from Western blotting using anti-penta-His antibody (Fig. 3, B and C). Again, analysis of the ATPase activity across the elution profile showed enzymatic activity, indicating that PM H⁺-ATPase was correctly reconstituted in the nanodiscs (Fig. 3D). Additionally, the ATPase activity confirmed the presence of residual aha2Δ73 in the flow through. Size exclusion chromatography after nickel affinity chromatography on both the flow-through and the eluate confirmed efficient separation of ND-AHA2 from empty nanodiscs; ND-AHA2 eluted in a major peak ahead of the position for empty nanodiscs (Fig. 4A). A small shoulder on the trailing edge of the peak for nickel affinity-purified nanodiscs indicated a small proportion of empty discs, possibly assembled from residual amounts of His₇-tagged MSP1D1 still present in the reconstitution mixture. SDS-PAGE analysis on fractions from size exclusion on purified ND-AHA2 (Fig. 4B) revealed a complete co-elution pattern of aha2Δ73 and MSP1D1(−) devoid of the His₇ tag, further confirming the correct assembly of PM H⁺-ATPase containing nanodiscs.

Analytical Characterization of PM H⁺-ATPase-containing Nanodiscs—To determine Stokes diameter of the phospholipid bilayer nanodiscs, the size exclusion chromatography column was calibrated using a standard set of proteins. Based on this calibration, empty nanodiscs and ND-AHA2 had overall Stokes diameters of 10.4 and 11.8 nm, respectively. The calculated Stokes diameter for empty nanodiscs are in reasonable agreement with earlier results on MSP1D1 assembled nanodiscs, reporting a Stokes diameter of 9.6 nm for nanodiscs assembled using dipalmitoyl-PC lipids and MSP1D1(−) (24, 25). As illustrated in Fig. 5D, the largest dimension on a model of ND-
AHA2 results in values of about 11 nm, consistent with the measured Stokes diameter from SEC analysis.

The dimension and homogeneity of empty nanodiscs and ND-AHA2 were further assayed from analysis of transmission electron microscopy images (Fig. 5, A1 and B1). This revealed most of the discs, whether full or empty, to exhibit diameters in the range of 10–11 nm (Fig. 5C) and to be lying individually on the carbon film. However, within the samples of ND-AHA2, we observed a number of paired or even stacked discs (Fig. 5, B2 and B3). Because such side view positioned paired discs were only found in samples from full discs, it is likely that this phenomenon was due to interaction between the inserted pumps and probably not the process of preparation.

Homogeneity of the assembled and affinity-purified nanodiscs was further investigated by BN-PAGE analysis. Under these conditions, empty discs migrated at \(2011011\) 146 kDa (Fig. 6A, lane 1), whereas ND-AHA2 migrated as a single band at \(361\) kDa (Fig. 6A, lane 2). Upon solubilization of ND-AHA2 by SDS, single bands at \(2011011\) 200 and \(2011011\) 20 kDa were observed corresponding to free PM H\(^{+}\)-ATPase and MSP (Fig. 6A, lane 4), whereas solubilized empty nanodiscs displayed only a single band for MSP at \(2011011\) 20 kDa (Fig. 6A, lane 5). Consistent with previous BN-PAGE analysis, purified DDM-solubilized PM H\(^{+}\)-ATPase was detected at \(2011011\) 200 kDa (Fig. 6A, lane 3). Based on simple geometrical assumptions when estimating the amount of lipids in the discs, the apparent molecular mass of empty nanodiscs and ND-AHA2 was estimated to be 129 and 213 kDa, respec-
The structure of aha2Δ73 is colored according to the different domains (7); the 10 transmembrane segments are brown, and the N, P, and A domains are red, blue, and yellow, respectively. The model was prepared using Visual Molecular Dynamics (62).

**PM H\textsuperscript{+}-ATPase Reconstitutes into Nanodiscs as a Monomer**—The MSP/aha2Δ73 stoichiometry was found to be 2:1 from densitometry analysis of SDS-polyacrylamide gels (supplemental Fig. S2). The Stokes diameters estimated from the protein standards are in agreement with the values found from SEC and transmission electron microscopy analysis, with ~9.7 nm ~11.6 nm for empty nanodiscs and ND-AHA2 respectively.

**Enzymatic Properties of Plant Plasma Membrane H\textsuperscript{+}-ATPase in Nanodiscs**—To ascertain that the PM H\textsuperscript{+}-ATPase activity of purified and reconstituted PM H\textsuperscript{+}-ATPase had maintained its enzymatic properties, we analyzed the ATPase activity of purified and reconstituted PM H\textsuperscript{+}-ATPase, cross-linking was performed using the amine-specific cross-linker DMS, previously shown to cross-link DDM-solubilized aha2Δ73 dimers (Fig. 1C). Cross-linking of empty nanodiscs resulted in two additional bands migrating to ~40 kDa (D1) and ~55 kDa (D2) in SDS-PAGE (Fig. 6B, lanes 2 and 3). This pattern is consistent with previous reports of cross-linked ApoAI in reconstituted high density lipoprotein particles, resulting in two distinct dimer forms (52). The two dimer forms of MSP1D1(−) were also observed from cross-linking of ND-AHA2 on both Coomassie-stained SDS-PAGE and Western blotting using an ApoA-1\textsubscript{C-term} antibody (Fig. 6C, lanes 4 and 4*), confirming a similar conformation of MSPs in full and empty nanodisc complexes. From cross-linking of ND-AHA2, three additional bands at ~115 kDa (C1), ~150 kDa (C2), and above the 170-kDa marker (C3) were observed in Coomassie-stained SDS-PAGE (Fig. 6C, lane 4 left panel). Only the bands at C1 and C2 are clearly visible from Western blotting using AHA2\textsubscript{N-} and ApoA-1\textsubscript{C-term} antibodies (Fig. 6C, lanes 4 and 4*, right panel). Comparison of C1, C2, and C3 to the dimer product of cross-linked DDM-solubilized aha2Δ73 (Fig. 6C, lane 2) reveals all to migrate to lower molecular weights than the apparent aha2Δ73 dimer. Based on these observations, we conclude that C1, C2, and C3 are products of cross-linking between the MSPs and aha2Δ73 and that aha2Δ73 is reconstituted as a monomer in the nanodiscs.
FIGURE 7. Vanadate sensitivity of ATP hydrolytic activity of aha2Δ73 reconstituted in vesicle (open circles) and nanodisc (filled circles). Data are representative of at least two independent experiments and expressed as percentage of control measured in the absence of vanadate. Straight lines were fitted to the experimental data points to estimate the IC50 values (5.8 μM for vesicles; 4.4 μM for nanodiscs). Inset, confirmation of proton pumping of vesicle-reconstituted aha2Δ73 using the proton-dependent fluorophore ACMA. Pumping was initiated by the addition of MgSO4 (peak 1) and the resulting proton gradient was dissipated by addition of carbonyl cyanide m-chlorophenylhydrazone (peak 2).

Reconstituted into Liposomes

H+-ATPase, reconstituted into liposomes with POPC/POPG (3:2), a specific ATPase activity in the range of 10.5 μmol P_i/min/mg was estimated. Kinetic analyses revealed Km (ATP) of ~180 and ~60 μM for lipid-activated and nanodisc-reconstituted PM H+-ATPase, respectively. These values are within the range of previous studies on C-terminally truncated deletion mutants, reporting values of 50–500 μM for aha2Δ77 in endoplasmic reticulum vesicles from yeast (39) and 76 ± 5 μM for lipid-activated aha2Δ73 (37). Taken together, our kinetic analysis demonstrated that reconstitution of PM H+-ATPase into nanodiscs did not compromise with its enzymatic properties.

It is an intrinsic property of the nanodisc system that transport of reconstituted transporters cannot be readily assayed. Thus, in contrast to reconstitution of PM H+-ATPase into liposome vesicles, where transported protons can be made to accumulate from the extravesicular medium into the vesicle lumen, both sides of the nanodisc membrane are freely accessible to the assay medium, and transported protons cannot be trapped and assayed in the same way. In P-type ATPases, transport of ligands from the cytoplasmic to the extra-cytoplasmic side of the membrane takes place during the transition from the E1 conformational state, which hydrolyzes ATP, to the E2 conformational state, more specifically between the phosphorylated E1P and E2P states (53). Vanadate is a transition state analog of inorganic orthophosphate, which inhibits P-type ATPases by binding specifically to the E2 conformation (53). The vanadate sensitivity of PM H+-ATPase was measured following reconstitution of the enzyme in vesicles as well as in nanodiscs (Fig. 7). Prior to the assay, it was ascertained that the H+-ATPase reconstituted into liposomes actually pumps protons (Fig. 7, inset). PM H+-ATPase reconstituted in both membrane types was found to exhibit the same vanadate sensitivity (Fig. 7). This confirms that PM H+-ATPase reconstituted in both experimental systems has a comparable conformational equilibrium and when reconstituted in both systems are capable of entering the E2 state of the catalytic cycle. Although not direct evidence for proton pumping, this strongly indicates that the H+-ATPase reconstituted into nanodiscs is an active proton pump like the enzyme reconstituted into liposomes.

Immobilization of PM H+-ATPase-containing Nanodiscs on Penta-His Surface—We further investigated the potential of nanodiscs for future SPR-mediated investigation of the PM H+-ATPase. Because direct immobilization of proteins and nonspecific cross-linking of the protein to surfaces might impair protein function (54), the samples are immobilized on CM5 sensor chips covalently modified with antibodies specifically recognizing His tags (penta-His antibody). A high density of penta-His antibody sensor chip surface. The arrows mark start and end of injection and start of regeneration, respectively. A, immobilization of a mixed sample of empty nanodiscs and ND-AHA2. Concentrations of 0, 0.5, 1, 5, 10, and 15 μg/ml mixed samples was captured on the surface, and dissociation was observed for 10 min before regeneration. B, capture of 10 μg/ml ND-AHA2 separated from empty nanodiscs (black). Red curve shows sample run of 10 μg/ml empty nanodiscs assembled from MSP1D1(−).

FIGURE 8. Capture of aha2Δ73 reconstituted in nanodiscs on penta-His antibody sensor chip surface. The arrows mark start and end of injection and start of regeneration, respectively. A, immobilization of a mixed sample of empty nanodiscs and ND-AHA2. Concentrations of 0, 0.5, 1, 5, 10, and 15 μg/ml mixed samples was captured on the surface, and dissociation was observed for 10 min before regeneration. B, capture of 10 μg/ml ND-AHA2 separated from empty nanodiscs (black). Red curve shows sample run of 10 μg/ml empty nanodiscs assembled from MSP1D1(−).
confirms that the observed immobilization is due to interaction with the penta-His antibody. The sample containing empty nanodiscs and ND-AHA2 assembled with MSP1D1 has two to three His tags per complex, with one His tag on the PM H⁺-ATPase and two His tags from the two MSPs. From the avidity effect, the presence of several His tags per complex can promote stable immobilization of the nanodiscs to the surface, although this approach does not allow us to conclude specific immobilization of ND-AHA2 to the surface. For this purpose we applied nickel affinity-purified ND-AHA2 assembled from MSP1D1(−), which confirmed the specific immobilization of ND-AHA2 through the His₆ tag on aha2Δ73.

DISCUSSION

Nanodisc-embedded Plasma Membrane H⁺-ATPase Is an Active Monomer—In this study, we demonstrate that the functional unit of the PM H⁺-ATPase is a monomer. To do this, we worked out a reconstitution procedure for the plant PM H⁺-ATPase at the single molecule level into nanodiscs. Expression of His-tagged PM H⁺-ATPase from a multicopy plasmid under the control of the strong constitutive promoter (PMA1) allowed for effective purification to homogeneity of an C-terminally truncated version of the PM H⁺-ATPase. To facilitate the formation of nanodiscs with single PM H⁺-ATPase monomers, a 4-fold excess of nanodiscs to PM H⁺-ATPase was applied during the reconstitution procedure. Under this condition, the purified, solubilized ATPases efficiently incorporated in nanodiscs. BN-PAGE analysis of ND-AHA2 revealed a single slowly migrating band confirming the homogeneity of the assembled and affinity-purified nanodiscs.

Despite the oligomeric state of the initially purified detergent-solubilized H⁺-ATPase, our data show that the pump reconstitutes in nanodiscs as a monomer. This conclusion is based on two independent approaches as follows. 1) Isolation of ND-AHA2 allowed us to estimate the MSP/H⁺-ATPase stoichiometry by densitometry analysis of SDS-polyacylamide gels. 2) Cross-linking experiments excluded the presence of dimeric H⁺-ATPase. Cross-linking experiments also verified a similar conformation of the MSPs in both ND-AHA2 and empty nanodiscs, with a cross-linking product of two distinct dimers in both cases (Fig. 6, B, C, D1, and D2). Furthermore, these experiments confirmed the close proximity of PM H⁺-ATPase and MSP in the ND-AHA2 complex, giving three different cross-linking complexes after incubation with the DMS cross-linker (Fig. 6C). The three distinct bands with equal spacing likely represent cross-linking products of PM H⁺-ATPase and one, two, and two in dimer D2 form of MSPs, as also verified from Western blotting against aha2ΔΔ73 and MSP.

Notably, nanodisc-reconstituted PM H⁺-ATPase displayed a robust ATPase activity, demonstrating that the pump monomer remains folded in an active conformation. This observation is in line with earlier studies on vesicle-reconstituted N. crassa PM H⁺-ATPases demonstrating that the pump monomer is likely to be the minimal functional unit (17). The vanadate sensitivity of the nanodisc-reconstituted PM H⁺-ATPase confirmed that it is able to alternate between the E1 conformation, which hydrolyzes ATP, to the E2 conformation, which binds vanadate. This indicates that the nanodisc-embedded PM H⁺-ATPase carries out the complete catalytic cycle, which is associated with proton pumping. Furthermore, the similar vanadate sensitivity of PM H⁺-ATPase reconstituted in vesicles and nanodiscs suggests that no significant change in conformational equilibrium is introduced when reconstituting the enzyme in nanodiscs as compared with vesicles, where the PM H⁺-ATPase is shown to pump protons.

Nanodiscs as a Tool to Study P-type ATPase Function—In vitro studies involving the H⁺-ATPases have so far been based on either detergent solubilization or membrane vesicles. The presence of detergent is required throughout purification, manipulation, assays, and structural studies to prevent aggregation. Detergents often perturb interactions and strip the membrane proteins of their endogenous lipids, which may be required to retain their functional state. Reconstitution into vesicles is a powerful model for transport studies and molecular characterization. However, drawbacks in using this system include the heterogeneous nature of the prepared vesicles, orientation of the proteins effectively giving rise to two populations exposed to different solvent phases, and the barrier of the membrane to soluble proteins in membrane-protein-protein interaction studies.

Nanodiscs provide a complementary system for studying membrane pumps under defined experimental conditions. We found that the Vₐₚₜ max values of nanodisc-reconstituted PM H⁺-ATPase were similar to those measured for asolectin-activated, DDM-solubilized PM H⁺-ATPase. However, we observed an ~10-fold higher affinity for ATP of aha2ΔΔ73 in nanodiscs compared with lipid-activated aha2ΔΔ73. This could be due to both the MSPs interacting directly with the PM H⁺-ATPase or the different lipid environment compared with lipid/DMM micelles. A recent study on empty nanodiscs using small angle x-ray scattering reported area per lipid headgroup values higher for POPC and lower for dilauroyl-PC in nanodiscs compared with free bilayers (55). Differences in mean bilayer height and phase transition temperature between nanodisc and lamellar dipalmitoyl-PC and dimyristoyl-PC have also been reported using a combined small angle x-ray scattering and differential scanning calorimetry analysis (56). Here, the increase in transition temperature and altered mean bilayer height was explained by the additional lateral pressure and structural perturbations from the MSP. Evidently, the variations in these parameters depend on the applied lipid, although it clearly demonstrates a different lipid environment in nanodiscs compared with other lipid systems, which could explain the different apparent affinity of ATP for PM H⁺-ATPase in nanodiscs compared with lipid-activated PM H⁺-ATPase. Natural lipid membranes consist of a large variety of lipids and proteins, with membrane proteins constituting up to 80% of the total weight of membranes (57). As a result, a large fraction of the lipids in a membrane are in contact with proteins. In this light, it could be speculated that the lipid bilayer in a nanodisc more accurately reflects the natural environment found in membranes, as compared with vesicles of pure lipids and lipid/detergent micelles (58). Naturally, the absence of a proton gradient in the nanodisc system is another important difference compared with vesicle-reconstituted PM H⁺-ATPases, which may influence the kinetics and activity.
**H⁺-ATPase Reconstituted into Nanodiscs**

**Immobilization of Nanodiscs to Study Regulatory Pump Interactions**—Surface-sensitive techniques allow for real time measurement of biological events and thereby provide thermodynamics and kinetics information on the interaction. For this purpose, SPR is one of the most used techniques. Based on changes of refractive indexes at the surface, SPR does not require any labeling of the proteins. A requirement for the use of SPR is the immobilization of functional proteins onto surfaces. Furthermore, the improved stability of isolated membrane proteins in nanodiscs compared with detergent is a considerable advantage for investigation by surface-sensitive techniques (59–61). Using both a mixture of empty nanodiscs and ND-AHA2 assembled with MSP1D1 and nickel affinity-purified ND-AHA2, we found stable and specific immobilization to the sensor chip. The stability of the immobilization observed in all cases and the possible regeneration of the surface after treatment at low pH (pH 2) makes thus the proposed strategy suitable for further monitoring protein-protein interactions by SPR.

**Conclusion**—We have demonstrated that the PM H⁺-ATPases can be embedded into lipid bilayer nanodiscs as a functional monomer. The purification and reconstitution procedures developed here should be useful for the analysis of PM H⁺-ATPases and other P-type ATPases at the molecular level. The ability to study these pumps embedded in a nanodisc rather than in detergent micelles or vesicles enables the use of a broad arsenal of biochemical and biophysical tools to quantitatively characterize the embedded protein and protein-protein interaction studies between the pump and its soluble interaction partners.

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