The Yeast Plasma Membrane ATP Binding Cassette (ABC) Transporter Aus1

PURIFICATION, CHARACTERIZATION, AND THE EFFECT OF LIPIDS ON ITS ACTIVITY‡

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Magdalena Marek§, Sigrid Millers†, Gabriele Schreiber†, David L. Daleke§, Gunnar Dittmar¶, Andreas Herrmann†, Peter Müller†, and Thomas Günther Pomorski†,‡

From the †Institute of Biology, Humboldt University of Berlin, Invalidenstrasse 42, 10115 Berlin, Germany, ‡Department of Biochemistry and Molecular Biology, Indiana University, Bloomington, Indiana 47405, §Max-Delbrück Center for Molecular Medicine, Robert-Rösse Strasse 10, 13125 Berlin, Germany, and ¶Center for Membrane Pumps in Cells and Disease-PUMPKIN, Danish National Research Foundation, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

The ATP binding cassette (ABC) transporter Aus1 is expressed under anaerobic growth conditions at the plasma membrane of the yeast Saccharomyces cerevisiae and is required for sterol uptake. These observations suggest that Aus1 promotes the translocation of sterols across membranes, but the precise transport mechanism has yet to be identified. In this study, an extraction and purification procedure was developed to characterize the Aus1 transporter. The detergent-solubilized protein was able to bind and hydrolyze ATP. Mutagenesis of the conserved lysine to methionine in the Walker A motif abolished ATP hydrolysis. Likewise, ATP hydrolysis was inhibited by classical inhibitors of ABC transporters. Upon reconstitution into proteoliposomes, the ATPase activity of Aus1 was specifically stimulated by phosphatidylserine (PS) in a stereoselective manner. We also found that Aus1-dependent sterol uptake, but not Aus1 expression and trafficking to the plasma membrane, was affected by changes in cellular PS levels. These results suggest a direct interaction between Aus1 and PS that is critical for the activity of the transporter.

Members of the ATP-binding cassette (ABC) family of proteins mediate the active transbiliar export or import of substrates coupled to the hydrolysis of ATP (1, 2). Most ABC family members are integral proteins that interact intimately with membrane lipids, and some of these transporters utilize phospholipids among their substrates (3, 4). Furthermore, a number of ABC transporters have been implicated in the transport of sterols. In humans, two half-size ABC transporters, ABCG5 (5) and ABCG8 (6), which are highly expressed in epithelial cells of the intestine and act as a heterodimer (7, 8), have been linked to the efflux of cholesterol into bile (9), whereas two other proteins, ABCG1 and ABCG4, are held responsible for transport of cholesterol into high density lipoprotein particles (10–12).

Studies on sterol influx in the yeast Saccharomyces cerevisiae identified two ABC transporters, Aus1 and Pdr11, that localize primarily to the plasma membrane and are required for sterol uptake under anaerobic conditions (13, 14) and in mutants that lack heme (15). Under these conditions, Saccharomyces cerevisiae becomes dependent on exogenously supplied sterols as sterols are essential for the cell, and their synthesis requires oxygen. Deletion of both Aus1 and Pdr11 essentially abolishes the uptake of sterols and impairs growth during anaerobiosis (13, 14). Both proteins belong to the ABCG subfamily and are full-size transporters with two membrane-embedded transmembrane domains (TMDs) and two cytoplasmic nucleotide binding folds (NBFs) (16). Members of this subfamily are unique in their domain architecture as they display a reverse topology, i.e. NBF1-TMD1-NBF2-TMD2. The NBFs of both Aus1 and Pdr11 contain all characteristic sequence motifs of ABC transporters. These include the Walker A and Walker B motifs (which are involved in ATP binding and hydrolysis) and the signature C sequence, the hallmark of the ABC family.

A major unresolved question concerns the precise nature of sterol transport mediated by Aus1 and Pdr11. It has been proposed that both ABC transporters may transport sterol directly out of the plasma membrane to a cytosolic acceptor, such as soluble sterol-binding proteins, or closely apposed membranes of the endoplasmic reticulum (14). Alternatively, they may indirectly facilitate sterol transport by catalyzing the transbilayer movement of other lipids as suggested for other ABC transporters (17, 18) or be required for the entry of external sterol into...
the plasma membrane (19). Thus, direct biochemical proof of their function and key features of their activity remain to be elucidated.

To enable functional analysis of the Aus1 transporter, in the present study, purification and reconstitution procedures were developed. The ATPase activity was characterized in terms of effects of inhibitors and requirements for lipids and sterol. We found that phosphatidylserine (PS) specifically stimulated Aus1 ATPase activity in a stereoselective manner and was required for Aus1-dependent sterol uptake in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—8-Azido-[α-32P]ATP (12.5 Ci/mmol) was purchased from ALT BioScience (Lexington, KY); [γ-32P]ATP was from Hartmann Analytic (Braunschweig, Germany). The detergent n-dodecyl-β-maltoside (DDM) was obtained from Glycon Biochemicals (Luckenwalde, Germany). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), sphingomyelin, and 7-nitrobenz-2-oxa-1,3-diazole (NBD) lipids including 25-NBD-cholesterol (25-[N-(NBD-methyl)-amino]-27-norcholesterol), 1-palmitoyl-2-NDHexanoyl-sn-glycero-3-phosphoserine (C6-NBD-PS), and 1-palmitoyl-2-NBD-lauroyl-sn-glycero-3-phosphoserine (C12-NBD-PS) were purchased from Avanti Polar Lipids (Alabaster, AL). The synthesis of POPS stereoisomers is described in the supplemental data. All other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise indicated.

**Media, Strains, and Growth Conditions**—All yeast strains and plasmids used in this study are described in the supplemental data. Yeast strains were grown at 30 °C in standard synthetic dextrose or galactose medium lacking uracil and when necessary supplemented with 20 μg/ml methionine and 20 μg/ml β-aminolevulinic acid. Medium supplemented with fatty acids and sterols contained 0.05% Tween 80 and 20 μg/ml cholesterol, respectively. For cultivation of PS synthesis-deficient mutants (cho1Δ), media were supplemented with 1 mM ethanolamine and 0.2 mM myo-inositol. Escherichia coli strain DH5α was used for all plasmid amplifications and isolations according to standard protocols (20).

**Lipid Uptake Assays**—Uptake of 25-NBD-cholesterol was analyzed in cells cultured for 16 h in minimal medium containing 0.05% Tween 80 and 20 μg/ml cholesterol mixture (cholesterol/25-NBD-cholesterol, 1:1, w/w). Before analysis by flow cytometry or confocal microscopy, cells were washed twice with ice-cold phosphate-buffered saline (PBS; 130 mM NaCl, 2.6 mM KCl, 7 mM Na2HPO4, 1.2 mM KH2PO4, pH 7.4) containing 0.05% (w/v) Nonidet P-40, and finally cells were resuspended in PBS. Uptake of C6-NBD-PS was analyzed as described before (21) with small modifications. Briefly, cells were grown to midlogarithmic phase (A600 ∼ 0.6–0.8) on standard synthetic dextrose medium and subsequently harvested by centrifugation (3,000 × g, 5 min, room temperature). Cells (5 × 107) were incubated in 250 μl of standard synthetic dextrose medium with 60 μM C6-NBD-PS for 30 min at 30 °C with periodic mixing. Prior to analysis by flow cytometry, cells were washed twice in ice-cold medium containing 3% (w/v) bovine serum albumin to extract C6-NBD-PS from the cell surface. Flow cytometry was performed on a BD Biosciences FACS analyzer equipped with an argon laser using CellQuest software. One microliter of 1 mg/ml propidium iodide in water was added to 107 cells in 1 ml of PBS just before flow cytometry analysis. Cells were analyzed without gating during the acquisition, and the data were analyzed by CytoLogic software. A histogram of the red fluorescence (propidium iodide) was used to set the gate that excluded dead cells from the analysis. Green fluorescence (C6-NBD-PS) of living cells was plotted on a histogram, and the geometric mean of the fluorescence intensity was calculated.

**Fluorescence Microscopy**—Confocal laser scanning microscopy was performed using an inverted Fluoview 1000 microscope (Olympus, Tokyo, Japan) and a 60× (numerical aperture, 1.35) oil immersion objective. Fluorescence of NBD was excited with a 488 nm argon laser and recorded between 500 and 530 nm. Red fluorescence protein (RFP) was excited using a 559 nm laser, and emission was recorded between 570 and 670 nm.

**Purification of Aus1**—The S. cerevisiae strain BJ1991 expressing FLAG-tagged Aus1 was grown at 30 °C in selective standard synthetic dextrose medium to an A600 of 1–1.5. Cells were harvested (3,000 × g, 5 min, 4 °C), taken up in selective standard synthetic galactose medium, and cultured at 30 °C to induce Aus1 expression. Cells were collected 16 h after induction unless otherwise indicated and lysed by vortexing five times for 1 min with acid-washed glass beads (10 g, 0.5 mm, Sigma) and 10 ml of ice-cold lysis buffer (100 mM KCl, 50 mM Tris-HCl, pH 7.4) containing protease inhibitor mixture (Roche Applied Science) and 1 mM phenylmethylsulfonyl fluoride. The lysate was clarified by centrifugation (500 × g, 10 min, 4 °C). Membranes were collected from the precleared lysates by centrifugation (60,000 × g, 45 min, 4 °C) and detergent-solubilized at a protein concentration of 1 mg/ml in immunoprecipitation buffer (100 mM KCl, 20% (w/v) glycerol, 50 mM Tris-HCl, pH 7.4) supplemented with 1% (w/v) DDM on an end-over-end rotator for 2 h at 4 °C. Insoluble material was removed by centrifugation (100,000 × g, 45 min, 4 °C), and the supernatant was incubated with 14 μl/ml anti-FLAG (M2) affinity resin for 16 h at 4 °C. The resin was washed three times for 10 min at 4 °C with immunoprecipitation buffer containing 0.05% (w/v) DDM. Proteins were eluted with 100 μg/ml FLAG peptide in elution buffer (immunoprecipitation buffer containing 0.05% (w/v) DDM). Protein was concentrated on Amicon Centricon filter devices (Millipore Corp., Bedford, MA) with molecular mass cutoff of 50 kDa. The eluate was analyzed by SDS-PAGE and Coomassie Blue staining. The protein bands of interest were excised, digested by trypsin, and analyzed by mass spectrometry as described in the supplemental data. Western blot analysis was performed with anti-FLAG M2 antibodies (1:5,000) and a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and visualized by enhanced chemiluminescence (ECL Plus kit, GE Healthcare). Blue native PAGE analysis was performed as described by Wittig et al. (22). Concentrations of purified Aus1 were determined by Coomassie Blue staining with a bovine serum albumin molecular weight standard via densitometry analysis using a Fuji FLA-3000 imaging system and AIDA Image Analyzer 3.24 software (Raytest, Straubenhardt,
Nucleotide Binding Assay—Nucleotide binding was measured by 8-azido-[α-32P]ATP photocross-linking experiments. Reactions were performed in a 96-well microtiter plate in a final volume of 25 μl/reaction. Purified wild-type or mutant Aus1 (about 2 μg of protein) was incubated for 5 min on ice with 8-azido-[α-32P]ATP (0.01–20 μM) in reaction buffer (100 mM KCl, 2.5 mM MgCl2, 50 mM Tris-HCl, pH 7.4). For competition experiments, 0.1 μM to 20 mM unlabeled ATP was included in the buffer. Subsequently, samples were irradiated with UV light (254 nm, 8 watts) for 5 min at 4 °C, separated by SDS-PAGE, Comassie Blue-stained, dried, and exposed to a phosphor screen. Samples were visualized with a Fuji FLA-3000 imaging system, and bands were quantified using AIDA Image Analyzer 3.24 software. Apparent Kd values for 8-azido-[α-32P]ATP were obtained from the best fit of the data to a hyperbolic curve using SigmaPlot software (Systat Software, Inc.) and the equation $Y = B_{max} X / (K_{d(azidoATP)} + X)$ where $B_{max}$ is the maximal binding, $X$ is the concentration of 8-azido nucleotide, and $K_{d(azidoATP)}$ is the concentration of the 8-azido-nucleotide required to reach half-maximal binding. The half-maximal inhibitory concentration (IC50) for ATP was derived by plotting labeling intensities corresponding to Aus1 as a function of unlabeled ATP concentrations. The $K_{d(azidoATP)}$ values and the IC50 values were used to calculate $K_{d(AP)}$ by applying the Cheng-Prussoff equation (23).

Reconstitution of Liposomes and Proteoliposomes—For protein reconstitution, lipids were dissolved in chloroform, and the solvent was evaporated. The lipid film (2.5 mg) was rehydrated in 1 ml of reconstitution buffer (150 mM KCl, 20 mM HEPES, pH 7.5). The resulting multilamellar liposomes were extruded 31 times through a polycarbonate filter of a pore size of 200 nm (Avanti Polar Lipids). The obtained unilamellar liposomes were destabilized by incubation with 1.5 mM DDM. Proteoliposomes were prepared by addition of purified protein to destabilized liposomes in a lipid to protein ratio of 20:1 (w/w) followed by a 15-min incubation at room temperature. Protein-free liposomes were prepared similarly by replacing purified protein with elution buffer. DDM was removed by incubation for 16 h at room temperature with 0.3 g/ml SM-2 Adsorbent Bio-Beads (Bio-Rad). The resulting proteoliposomes were collected by centrifugation (100,000 × g, 1 h, 4 °C), resuspended in reconstitution buffer, and used for assays immediately. For density flotation, a total of 250 μl of proteoliposomes was mixed with an equal volume of 80% Nycodenz in an 11 × 60-mm centrifuge tube (Beckman) and overlaid with 0.5 ml of 30%, 0.5 ml of 20%, 0.5 ml of 10%, 0.5 ml of 5%, and 0.5 ml of 2.5% (w/v) Nycodenz and 0.5 ml of reconstitution buffer. After centrifugation (130,000 × g, 4 h, 4 °C), 14 fractions (0.25 ml) were collected from the top of the centrifuge tubes and analyzed for ATPase fluorescence and total phosphate determination (24).

Collisional Quenching Assay—To elucidate the orientation of reconstituted Aus1-RFP, collisional quenching of RFP fluorescence with CuCl2 was performed (25). Vesicles in the absence and presence of 0.5% (w/v) Triton X-100 were titrated with CuCl2 (0–30 μM as indicated) until RFP fluorescence intensities did not decrease further. Parallel samples were measured in which MgCl2 was used instead of CuCl2, showing that magnesium had no detectable effect on RFP fluorescence. Data were corrected for volume changes and scattering. The fraction of RFP that is accessible to the quencher was calculated as $(F_0 - F_{Cu})/(F_0 - F_o) \times 100$ where $F_o$ is the initial fluorescence of vesicles in buffer without CuCl2, $F_{Cu}$ is the fluorescence plateau value of vesicles titrated with CuCl2, and $F_0$ is the fluorescence plateau value of Triton X-100-permeabilized vesicles titrated with CuCl2.

ATPase Assay—Purified Aus1 (2–10 μg) was mixed with 45 μl of reconstitution buffer containing 2 mM dithiothreitol and incubated for 20 min at 23 °C for protein activation. ATPase activity was analyzed by measuring the release of inorganic phosphate (Pi) using two methods: (i) a spectrophotometric assay following the manufacturer’s instructions (EnzChek® phosphate assay kit, Invitrogen) and (ii) an assay with [γ-32P]ATP as described by Gorbulev et al. (26). Briefly, for the [γ-32P]ATP assay, 5 μl of ATP mixture (1 mM ATP, 5 mM MgCl2, 2 μCi of [γ-32P]ATP) was added to the mixture, and the reaction was carried out for 40 min at 27 °C. The reaction was stopped by placing samples on ice and the addition of 1.5 ml of reagent A (10 mM ammonium molybdate in 1 n HCl), 15 μl of 20 mM H3PO4, and 3 ml of reagent B (isobutanol, cyclohexane, acetone, and reagent A in a ratio of 5:5:1:0.1, v/v). The mixture was mixed vigorously for 30 s. After phase separation, 1 ml of the organic phase was mixed with scintillation fluid (Ultima Gold XR, PerkinElmer Life Sciences), and the release of inorganic phosphate was determined by β-counting (Packard liquid scintillation analyzer). A similar protocol was used to determine the ATPase activity of reconstituted Aus1. Inhibition was assayed in mixtures containing ATP mixture supplemented with either 1 mM orthovanadate, 1 mM BeSO4 and 5 mM NaF (beryllium fluoride), or 1 mM AlF3. Orthovanadate solutions (100 mM) were prepared from Na3VO4 (Fisher Scientific GmbH) at pH 10 and boiled for 15 min before each use to break down polymeric species (27).

RESULTS

Cloning and Expression of Aus1—To facilitate purification of Aus1 for biochemical analysis, the ABC transporter was tagged at the N terminus with a FLAG epitope and expressed from a multicopy vector under the control of the strong inducible galactose promoter (GAL10). In addition, a plasmid was constructed to allow for expression of FLAG-tagged Aus1 fused at the C terminus with the monomeric RFP. To study the functionality of tagged proteins, the resulting constructs or the vector alone was used to transform the sterol uptake-deficient mutant hem1Δaus1Δpdr11Δ. This mutant grows normally on medium containing δ-aminolevulinic acid, the enzymatic product of the first step in heme biosynthesis catalyzed by aminolevulinic synthase (28), but cannot grow on medium containing sterols (ergosterol or cholesterol) due to the lack of Aus1 and Pdr11 (13). As shown in Fig. 1A, both tagged proteins retained their activity in vivo because they could restore the growth defect of the triple mutant hem1Δaus1Δpdr11Δ on cholesterol-containing medium. Likewise, overexpressed Aus1 protein supported the uptake of a fluorescently labeled cholesterol derivative (25-NBD-cholesterol) in hem1Δ and
hem1Δaus1Δpdr11Δ cells as revealed by flow cytometry and fluorescence microscopy (Fig. 1A and data not shown). Inactive mutants of tagged Aus1 were generated by introducing a lysine to methionine substitution in the Walker A region of NBF2 (Aus1K788M) which is known to block the ATPase activity of ABC proteins (29). Upon galactose induction, Aus1 mutant proteins were expressed but as expected were unable to restore the growth defect of the triple mutant hem1Δaus1Δpdr11Δ on sterol-containing medium (Fig. 1A).

Fluorescence microscopy revealed that a significant portion of Aus1K788M-RFP mutant reaches the plasma membrane (Fig. 1C).

Optimization of Expression and Solubilization of Aus1—The plasmids described above were used to transform the protease-deficient S. cerevisiae strain BJ1991. Individual colonies were assayed for Aus1 expression upon galactose induction by immunoblotting of total membrane preparations. Several individual colonies expressing high amounts of Aus1 were isolated for further analysis. Aus1 expression was found to be pH-sensitive and time-dependent, reaching optimal levels at pH 6–7 and 12–16 h after induction. To determine optimal solubilization conditions, membranes prepared from S. cerevisiae strain BJ1991 expressing Aus1 were exposed to a panel of four different conditions using DDM or Triton X-100 as detergents. DDM at 1% showed the highest solubilization efficiency and was used for all purification steps.

For Aus1 and Aus1-RFP purification, DDM-solubilized fractions were subjected to anti-FLAG affinity chromatography, and subsequently bound proteins were eluted by treatment with a buffer containing FLAG peptide. Separation of the elution fraction and staining with Coomassie Blue showed a single band of about 160 kDa consistent with the molecular mass of Aus1 (Fig. 2A, lane 1). Densitometric analysis of the gels of purified Aus1 revealed at least a 90% degree of protein purity. Western blotting (Fig. 2A, lane 2) and analysis by mass spectrometry (supplemental Table S3) confirmed the protein to be...
Aus1. Additionally, mass spectrometric analysis revealed that based on the number of peptides recovered Aus1 was the most abundant protein in the eluate (supplemental Table S4).

A typical purification procedure yielded about 70 μg of protein/1 g of wet cell pellet. To evaluate the oligomeric state of purified Aus1 and Aus1-RFP, the proteins were subjected to blue native PAGE analysis. The proteins migrated as single bands according to their monomeric size of ~160 and 185 kDa, respectively (Fig. 2B, lanes 1 and 2).

**Purified Aus1 Binds ATP and Displays ATPase Activity**—Transport functions mediated by members of the ABC transporter family require ATP binding and hydrolysis. To examine the interaction of Aus1 with ATP, the detergent-solubilized, purified protein was incubated at 4 °C with 8-azido-[α-32P]ATP, and the protein complex was exposed to UV light followed by analysis by SDS-PAGE. The SDS-PAGE gels were subjected to autoradiography for detection of 8-azido-[α-32P]ATP labeling and to Coomassie Blue staining for determination of protein content (Fig. 3A). A major band of about 160 kDa consistent with the molecular mass of Aus1 was identified by Coomassie Blue staining and found to be photoaffinity-labeled by 8-azido-[α-32P]ATP. Labeling of Aus1 by 8-azido-[α-32P]ATP required magnesium and was strongly inhibited by addition of EDTA (1 mM) or excess cold ATP (10 mM) or pre-treatment with 1 mM N-ethylmaleimide, a covalent SH group reagent (Fig. 3A). Taken together, these data indicate that the protein had retained its ability to bind ATP through the purification process. The apparent affinity constant for 8-azido-ATP was determined to be 0.12 μM (Fig. 3B). Photoaffinity labeling of Aus1 by 8-azido-[α-32P]ATP was inhibited by ATP with an IC50 value of 3.6 μM (Fig. 3C). Based on the apparent affinity of 8-azido-ATP, a dissociation constant for ATP of 0.2 μM was estimated.

Next, we analyzed the ATPase activity of the purified protein in detergent-containing buffer. Using a spectrophotometric assay for released Pi, we found that ATP was hydrolyzed by the protein in a time-dependent manner at 27 °C as revealed by the linear increase of released phosphate ions during the first 30 min. From that, a specific ATPase activity of the purified, detergent-solubilized Aus1 in the range of about 56 nmol of ATP/min/mg of protein was estimated. Using radiolabeled ATP, we next determined the ATPase activity in the presence of several ATPase inhibitors (Table 1). We found that the ATPase activity was not affected in the presence of ouabain or azide but was strongly inhibited by the classical inhibitors of ABC proteins, vanadate, AlF3, and beryllium fluoride. The calcium channel blocker verapamil, which has been reported to modulate the ATPase activity of the human multidrug transporter P-glycoprotein (30), hardly affected Aus1 ATPase activity at low concentrations (10 and 100 μM) but inhibited it at higher concentration (1 mM). To confirm that the observed ATPase activity is Aus1-specific, the ATPase-inactive mutant Aus1K788M was analyzed; it was solubilized and purified identically to the wild-type protein in similar yields (data not shown). The purified mutant protein showed very low background ATPase activity (2.9 ± 0.6% relative to wild type; three determinations). We conclude that the observed ATPase activity is Aus1-dependent.

**Reconstitution of Aus1 into Vesicles**—The membrane-embedded ABC transporter was characterized by reconstituting Aus1 into preformed detergent-distabilized liposomes. To verify successful reconstitution, we reconstituted Aus1-RFP and floated the resulting vesicles in a Nycodenz gradient. Fig. 4A


**Purification and ATPase Activity of Aus1**

**TABLE 1**

| Compound          | Final concentration | ATPase activity |
|-------------------|---------------------|-----------------|
|                   | µM                  | %               |
| Control           | 100.00              |                 |
| Vanadate          | 1                   | 2.64 ± 0.03     |
| BeSO₄/NaF (BeFx⁺) | 1/5                 | 21.63 ± 0.18    |
| AIF₂             | 1                   | 14.17 ± 0.06    |
| Sodium azide      | 5                   | 108.04 ± 0.51   |
| Ouabain           | 5                   | 108.12 ± 0.38   |
| Verapamil         | 1                   | 4.16 ± 0.30     |
| Verapamil         | 0.1                 | 86.74 ± 6.67    |
| Verapamil         | 0.01                | 111.07 ± 6.77   |

* ATP hydrolysis of the purified detergent-solubilized transporter was assayed by determination of the total Pi release during 40 min at 27 °C using [γ³²P]ATP. Results are the means ± S.D. of three independent determinations relative to the value obtained for the purified detergent-solubilized protein in the absence of inhibitors (control).

* Beryllium fluoride.

**FIGURE 4. Reconstitution of Aus1 into vesicles.** A, flotation of Aus1-RFP proteoliposomes in a Nycodenz gradient. Purified Aus1-RFP was reconstituted into preformed detergent-destabilized liposomes as described under "Experimental Procedures." Aus1-RFP-containing liposomes were applied to a Nycodenz gradient and centrifuged, and the obtained fractions were analyzed for RFP fluorescence (dashed line) and phospholipid content (solid line). Reconstitution was evident from co-migration of phospholipid and Aus1-RFP. A control sample containing only purified, solubilized Aus1 in the absence of lipids was mock-treated in 0.005% n-dodecylmaltoside-containing buffer at the same temperatures as the reconstitution samples (dotted line). B, Coomassie Blue-stained 8% SDS-PAGE gel of purified, solubilized Aus1-RFP (Control) and Aus1-RFP reconstituted in proteoliposomes composed of different lipids. PC, POPC only; PS, POPC/POPS (7:3). Molar mass markers are indicated on the left. C, membrane orientation of reconstituted Aus1-RFP. Vesicles in the absence and presence of 0.5% (w/v) Triton X-100 were titrated with CuCl₂ until saturation, and the accessible fraction of RFP was determined as described under "Experimental Procedures." A representative experiment from three independent determinations is shown. a.u., arbitrary units.

shows that Aus1-RFP floated with the vesicles to the top of the gradient, whereas purified, nonreconstituted protein did not float. These data suggest that Aus1 was successfully reconstituted into vesicles. SDS-PAGE analysis of proteoliposome samples indicated that Aus1 was not degraded during the reconstitution process regardless of the lipid composition of the proteoliposomes (Fig. 4B). The orientation of Aus1 after insertion into the proteoliposomes was determined by a collisional quenching approach in which membrane-impermeant Cu²⁺ ions were used to quench the fluorescence of RFP, which is accessible on the outside of the vesicles. Comparison of the quenching data of intact and detergent-permeabilized vesicles revealed that about 50–60% of Aus1 was inserted into POPC vesicles with the NBFs facing outward (Fig. 4C). Proteoliposomes composed of POPC/POPS (7:3) and POPC/POPE (7:3) were also analyzed for Aus1 orientation. Here, about 70–80% of Aus1 was inserted into these vesicles with the NBFs facing outward (data not shown). However, we never observed an exclusive asymmetric orientation of Aus1 toward one side of the vesicles.

**ATPase Activity of Reconstituted Aus1 Is Stimulated by Phosphatidylserine**—Next, we assessed the ATPase activity of Aus1 reconstituted in proteoliposomes of different lipid compositions using radiolabeled ATP. When Aus1 was reconstituted into POPC, POPC/POPE, or cholesterol/sphingomyelin proteoliposomes, we observed a decrease in its ATPase activity in comparison with the purified, detergent-solubilized protein, whereas in POPC/phosphatidylinositol and POPC/cholesterol vesicles, Aus1 ATPase activity was similar to that of the purified, detergent-solubilized protein (Fig. 5A). In contrast, reconstitution of Aus1 in POPC/POPS proteoliposomes resulted in a strong stimulation of the ATPase activity, showing a 5–8-fold increase in the activity of vesicles containing 30 mol % of PS. This multifold increase in ATPase activity cannot be explained by the slight differences observed in the orientation of Aus1 in the vesicle composed of different lipid compositions. Thus, the presence of PS in the liposomes must specifically promote the ATPase activity of Aus1.

To provide further insight into the specific requirement of Aus1 for PS, we explored the ability of PS stereoisomers to stimulate ATPase activity of Aus1 (Fig. 5B). Maximal activation occurred in the presence of the naturally occurring 1-palmitoyl-2-oleoyl-sn-phosphatidyl-l-serine (1,2-sn-POP-L-S). Altering the stereochemistry in the serine head group (1-palmitoyl-2-oleoyl-sn-phosphatidyl-d-serine (1,2-sn-POP-D-S) or in the glycerol backbone (2,3-sn-POP-L-S and 2,3-sn-POP-D-S) caused a decrease in ATPase activity. Maximum activation of Aus1 in the presence of natural PS and the ability of the enzyme to discriminate between various PS stereoisomers indicate that the activation results from the association of PS with a specific binding site on the enzyme.

**PS Is Required for Aus1-dependent Sterol Uptake in Living Cells**—The specific stimulation of Aus1 ATPase activity by PS suggests that this phospholipid might be a substrate for the ABC transporter. Therefore, we examined the uptake of C₆-NBD-PS by flow cytometry in hem1Δ cells containing or lacking both AUS1 and PDR11. As shown in Fig. 6A, no significant differences in the internalization of C₆-NBD-PS were observed between cells containing or lacking these two ABC proteins. C₆-NBD-PS has been shown previously to be predominantly internalized by transbilayer transport across the plasma membrane, resulting in intracellular labeling of various organelles (31). This result was confirmed for both hem1Δ cells containing and lacking AUS1 and PDR11 by examining the intra-
cellular localization of C₆-NBD-PS by fluorescence microscopy (Fig. 6B). As a control, we verified that NBD-PS analogs were able to stimulate the ATPase activity of reconstituted Aus1 to a similar extent as the natural PS (Fig. 5A). Together, these results suggest that PS may affect the transport activity of Aus1 rather than serving as a substrate for Aus1.

To further test this hypothesis, we generated hem1Δ cells lacking PS by disrupting the CHO1 gene encoding PS synthase (32) and examined sterol uptake. Both hem1Δ and hem1Δaus1Δpdr11Δ cells were incubated with 25-NBD-cholesterol for 16 h followed by flow cytometry analysis. As shown in Fig. 6C, in PS-deficient cells, accumulation of 25-NBD-choles-
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Sterol was strongly reduced by about 60% when compared with that in isogenic hem1Δ cells, consistent with a reduction in Aus1 transport activity. For comparison, the low uptake ability of hem1Δaus1Δpdr11Δ, cho1Δ, and the parental strain (W303) are shown. Furthermore, hem1Δcho1Δ cells grew on medium containing δ-aminolevulinic acid but were unable to grow on medium containing cholesterol (supplemental Fig. S1). Notably, Aus1 expression and trafficking were not affected in PS-deficient cells as judged from microscopic imaging of Aus1-RFP expressed from its endogenous promoter (Fig. 6D). These findings support the view that PS is critical for and regulates the activity of Aus1.

DISCUSSION

In this study, we describe for the first time a successful purification protocol and the biochemical characterization of the yeast putative sterol transporter Aus1. Expression of a FLAG-tagged version of Aus1 from a multicopy plasmid under the control of the strong inducible promoter (GAL10) allowed for effective purification to homogeneity in yields high enough for subsequent biochemical studies. Further modification of Aus1 by addition of RFP fused at its C terminus retained the functionality of the ABC transporter and facilitated the analysis of its expression, purification, and reconstitution into proteoliposomes. Similar approaches based on the use of the green fluorescent protein have proven very successful to rapidly test expressibility, stability, and monodispersity of various membrane proteins as well as to optimize their expression and purification (33, 34). In addition to the advantages of a fluorescent marker, RFP could be used as a tool to determine the orientation of protein insertion into proteoliposomes. For this, we used a collisional quenching approach (25) in which membrane-impermeant Cu²⁺ ions were used to selectively quench the fluorescence of RFP moieties localized on the outside of the vesicles. Under our experimental conditions, Aus1 was inserted into the liposomes with about 50–80% of the inserted transporter having its ATP-binding sites accessible to substrate binding and hydrolysis. Different lipid compositions of liposomes yielded only slight deviations from a random orientation distribution of Aus1. The quenching approach applied here should also be useful for the reconstitution analysis of other membrane proteins.

Purified, solubilized Aus1 was able to bind ATP in the presence of Mg²⁺ and exhibited an orthovanadate-, beryllium fluoride-, and AlF₃ sensitive ATPase activity even before reconstitution in liposomes, consistent with the properties of other purified ABC transporters, such as the human multidrug transporter P-glycoprotein (35, 36). ATPase activity was also effectively blocked at a higher concentration (1 mM) of verapamil. Conceivably, binding of numerous verapamil molecules to Aus1 might start to induce structural perturbation in the protein and block the transport cycle. A specific activity of the purified protein of 56 nmol of ATP/min/mg of protein was determined; this is within the range of values reported for a number of eukaryotic ABC transporters that have been purified and biochemically characterized, such as Ste6p, ABCR, transporter associated with antigen processing, and ABCA1 (26, 37–39). Upon reconstitution into proteoliposomes of different lipid composition, Aus1 ATPase activity was reduced by phosphatidylcholine and phosphatidylethanolamine but was specifically stimulated severalfold in the presence of PS. This stimulation cannot simply be explained by the presence of an anionic (charged) lipid because the anionic lipid phosphatidylinositol failed to enhance Aus1 ATPase activity substantially. Furthermore, the stimulation of Aus1 ATPase activity by PS was stereoselective; the natural stereoisomer of PS was more potent than its enantiomers in enhancing the ATPase activity. Although other lipids may also be important for Aus1 function, our data suggest that the direct and stereospecific interaction between the phospholipid PS and Aus1 regulates the activity of the transporter.

Many ABC transporters have been shown to possess intrinsic ATPase activity that is stimulated in the presence of transported substrates. The best investigated example is the mammalian Mdr1 (P-glycoprotein), which possesses an ATPase activity stimulated by various drugs that are known to be transported (40). Hence, the specific stimulation of Aus1 ATPase activity by PS suggests that this phospholipid might be the primary substrate for the ABC transporter, and translocating PS across the plasma membrane could be the physiological function of Aus1. However, our present results rule out this possibility; the presence or absence of Aus1 had no effect on the internalization and localization of NBD-PS and other phospholipid analogs across the yeast plasma membrane. Instead, we found that the Aus1-dependent sterol uptake was drastically reduced in PS-deficient cells, whereas expression and localization of the protein were not altered under these conditions. These in vivo data support a model in which PS is required for the full activity of the transporter to drive sterol uptake.

Biochemical and structural information obtained in recent years has highlighted an intimate relationship between membrane proteins and lipids that is important for full functional and structural integrity of the protein. For example, the activity of the yeast cytochrome bc₁ complex requires cardiolipin, and specific lipid-binding sites have been identified in x-ray structures of the protein complex (41, 42). Changes in phosphoethanolamine levels have recently been shown to affect the activity of the vacuolar membrane-localized ABC transporter Ycf1 (43). Similarly, Aus1 activity may be regulated by the PS levels in the cell. As this lipid is enriched along the secretory pathway, constituting ~13% of the glycerophospholipids in late secretory vesicles and ~34% in the plasma membrane, respectively (44), Aus1 activity would increase as it transits the secretory pathway en route to its functional residence in the plasma membrane. Notably, a requirement for PS has also been reported for the activity of the tryptophan transporter in the plasma membrane of S. cerevisiae (45).

The specificity of lipid-stimulated Aus1 ATPase activity is reminiscent of the activation of lipid-transporting ATPases from the P₇₋ ATPase family. Like Aus1, the ATPase activity of Atp8a1 (46) and Atp8a2 (47) are selectively activated by PS, and at least for Atp8a1, the enantiomeric specificity is qualitatively similar; enzyme activation is greatest in the presence of the L-serine stereoisomers. These data indicate that the binding sites for PS on these very
different classes of proteins may be similar or may reflect some other common features of their interactions with lipids.

How Aus1 facilitates sterol uptake remains to be clarified. Notably, we did not observe stimulation of ATPase activity of reconstituted Aus1 by cholesterol. The lack of ATPase stimulation by sterols has also been reported for other purified ABC transporters involved in sterol transport (8, 39). Conceivably, Aus1 function may require additional proteins either serving as cholesterol acceptors or donors. For example, the cell wall protein Dan1 has been shown to directly affect sterol uptake (13, 48) and may serve as a substrate-binding protein presenting sterol molecules to Aus1. The purification and reconstitution procedures developed here should be useful for unraveling the precise mechanism of Aus1-catalyzed sterol transport.

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