Purification and ATP Hydrolysis of the Putative Cholesterol Transporters ABCG5 and ABCG8†

Zhanling Wang,‡ Lindsay D. Stalcup,‡ Brandy J. Harvey,‡ Joachim Weber,§ Maja Chloupkova,|| Mark E. Dumont,⊥ Michael Dean,@ and Ina L. Urbatsch*,‡

Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79430, Department of Pediatrics, Oregon Health and Science University, Portland, Oregon 97239, Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, New York 14642, and Laboratory of Genomic Diversity, National Cancer Institute, Frederick, Maryland 21702

Received April 24, 2006; Revised Manuscript Received June 10, 2006

ABSTRACT: Mutations in the ATP-binding cassette (ABC) transporters ABCG5 and ABCG8 lead to sitosterolemia, a disorder characterized by sterol accumulation and premature atherosclerosis. ABCG5 and ABCG8 are both half-size transporters that have been proposed to function as heterodimers in vivo. We have expressed the recombinant human *ABCG5* and *ABCG8* genes in the yeast *Pichia pastoris* and purified the proteins to near homogeneity. Purified ABCG5 and ABCG8 had very low ATPase activities (<5 nmol min^{-1} mg^{-1}), suggesting that expression of ABCG5 or ABCG8 alone yielded nonfunctional transporters. Coexpression of the two genes in *P. pastoris* greatly increased the yield of pure proteins, indicating that the two transporters stabilize each other during expression and purification. Copurified ABCG5/G8 displayed low but significant ATPase activity with a V_{max} of ~15 nmol min^{-1} mg^{-1}. The ATPase activity was not stimulated by sterols. The catalytic activity of copurified ABCG5/G8 was characterized in detail, demonstrating low affinity for MgATP, a preference for Mg as a metal cofactor and ATP as a hydrolyzed substrate, and a pH optimum near 8.0. AlFx and BeFx inhibited MgATP hydrolysis by specific trapping of nucleotides in the ABCG5/G8 proteins. Furthermore, ABCG5/G8 eluted as a dimer on gel filtration columns. The data suggest that the hetero-dimer is the catalytically active species, and likely the active species in vivo.

Mutations in the *ABCG5* and *ABCG8* genes have been linked to sitosterolemia, a disease characterized by abnormal accumulation of plant sterols (e.g. sitosterol) in blood and various tissues (*1*−*3*). Patients with sitosterolemia exhibit increased absorption and decreased excretion of dietary sterols, and develop sterol deposits in skin, tendons (xanthomas), and arteries, leading to accelerated arteriosclerosis and ultimately premature coronary heart disease (*4*−*6*). Sitosterolemia is a rare disorder (*7*, *8*); yet, elucidation of the genetic defects in *ABCG5* and *ABCG8* has provided major new insights into the absorption and excretion of sterols (*9*−*13*).

The *ABCG5* and *ABCG8* gene products are normally expressed in intestines and liver, where they are localized to the brush border of enterocytes and to the apical surface of hepatocytes (*1*, *14*, *15*). Here, they are thought to reduce uptake and increase excretion of dietary sterols, respectively, by pumping the sterols into the lumen (*10*−*13*, *16*). In the lumen, the sterols are likely to be captured by phosphatidylcholine (PC) and bile acid micelles to keep them in solution for excretion (*17*). While it appears that these putative transporters are responsible primarily for export of plant sterols, accumulating evidence suggests that they may have a similar function in the transport of cholesterol. (i) Sitosterolemic patients are often hypercholesterolemic (*5*, *6*). (ii) Disruption of the *Abcg5* and *Abcg8* genes in mice results in a profound reduction of cholesterol in the bile, and plasma cholesterol levels are much more responsive to changes in dietary cholesterol than in control mice (*18*−*20*). (iii) Overexpression of human ABCG5 and ABCG8 in these knockout mice led to reduced absorption and increased secretion of dietary cholesterol (*21*−*23*). These data suggest that cholesterol, as well as plant sterols, is transported by ABCG5 and ABCG8. Thus, ABCG5 and ABCG8 may be important in cholesterol homeostasis, which is a major concern in arteriosclerosis.

ABCG5 and ABCG8 belong to the large family of ATP-binding cassette (ABC) transporters (*24*). These membrane-spanning transport proteins generally consist of two transmembrane domains (TMs)[1] that harbor the translocation sites for a specific substrate and two cytoplasmic nucleotide binding domains (NBDs) that power the transport reaction by hydrolyzing ATP (*25*). ABCG5 and ABCG8 are both

† This work was supported by the American Heart Association, Texas Affiliate 0465130Y, by the South Plains Foundation (Lubbock, TX), and by start-up funds of the Texas Tech University Health Sciences Center to I.L.U.

* To whom correspondence should be addressed: Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430-6540. Phone: (806) 743-1192. Fax: (806) 743-2990. E-mail: ina.urbatsch@ttuhsc.edu.

‡ Texas Tech University Health Sciences Center.

§ Texas Tech University.

|| Oregon Health and Science University.

⊥ University of Rochester Medical Center.

@ National Cancer Institute.

10.1021/bi0608055 CCC: $33.50
Published on Web 07/25/2006

half-size transporters with a single TM and a single NBD, and therefore, they are expected to function as homo- or heterodimers. Several types of experimental evidence support the proposal that ABCG5 and ABCG8 function as heterodimers. First, mutations in either gene cause sitosterolemia, and the two genes are arranged in a head-to-head orientation with common regulatory elements (*1*, *3*, *26*). They are coexpressed predominantly in the liver and small intestine (*1*, *15*). Furthermore, coexpression of the two proteins is required for proper trafficking out of the endoplasmic reticulum to the plasma membrane in cultured cells, and missense mutations in either gene disrupt trafficking to the cell surface (*27*, *28*). Finally, expression of both ABCG5 and ABCG8 is obligate for protein trafficking and biliary cholesterol excretion in transgenic mice (*14*). Because of the requirement for coexpression for correct trafficking, it has been difficult to study the function of the individual transporters in vivo.

In this study, we exploited the yeast *Pichia pastoris* for expression of the recombinant human *ABCG5* and *ABCG8* genes and purified the proteins to near homogeneity. We determined the ATPase activities of the individual transporters and of coexpressed and copurified ABCG5/G8. The ATPase activities were characterized in terms of lipid requirements, effects of sterols and inhibitors, and nucleotide binding parameters. Finally, the oligomeric status of the different proteins was analyzed with respect to their ATPase function.

EXPERIMENTAL PROCEDURES

Materials. FosCholine-14, *n*-tetradecylphosphatidylcholine (FC-14), and other detergents were obtained from Anatrace. Ni^{2+}−NTA agarose resin was from Qiagen, and calmodulin affinity resin was from Stratagene. Lyso-PC, *Escherichia coli* lipids, liver lipids, and PC lipids from liver and egg extracts were from Avanti (all acetone/ether-precipitated preparations). Asolectin (crude soybean phospholipid) was from Sigma. 8-Azido[α^{32}P]ATP was from Affinity Labeling Technologies, Inc. All other chemicals were at least p.a. grade from Fisher Scientific Inc.

Cloning and Expression of the Human ABCG5 and ABCG8 Proteins. cDNAs encoding full-length human *ABCG5* and *ABCG8* genes were obtained from A. Remaley (National Institutes of Health, Bethesda, MD). The ORFs were amplified by PCR and cloned under the control of the strong, methanol-inducible promoter *AOX I* in expression vector pSGP18 generating pSGP18-ABCG5 and pSGP18-ABCG8. This vector is a derivative of the *P. pastoris* pPICZ vector (Invitrogen) that was redesigned for ligation-independent cloning (M. Dumont et al., unpublished results). It contains in-frame sequences encoding a C-terminal 3C-protease cleavage peptide followed by two affinity purification tags, a calmodulin binding peptide tag (CBP), and an RGS-His_6 tag. The integrity of the individual ORFs was confirmed by DNA sequencing. pSGP18-ABCG5 and pSGP18-ABCG8 were transformed into *P. pastoris* strain KM71 by electroporation following standard procedures (Invitrogen Manual). Transformants were plated on YPDS containing 100, 500, and 1000 μg/mL Zeocin to select for single-copy and multicopy integrations. Several transformants from each plate were grown and induced in 10 mL cultures as previously described (*29*, *30*) with inclusion of 0.004% histidine in the media. "Rapid Membranes" were prepared (*30*), and 15 μg of microsomal membranes was resolved on 10% SDS gels for expression analysis on Western blots. For coexpression, equal amounts of pSGP18-ABCG5 and pSGP18-ABCG8 cDNAs were cotransformed into KM71; selection and screening followed the same procedures that were used for the individual genes.

Growth of Fermentor Cultures and Preparation of Microsomes. The fermentation of *P. pastoris* cultures was carried out as described previously (*30*), except that the PMT1 salts were reduced to 4.25 mL/L in the glycerol feed and methanol feed and the induction time in methanol was reduced to 20 h. From a 5 L fermentor culture, 1.3−1.5 kg of cells was harvested, washed with distilled water, resuspended at 0.5 g of wet cells/mL in mPIB buffer [0.3 M sucrose, 300 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 2 mM DTT, and 100 mM 6-aminohexanoic acid], and frozen at −80 °C in 100 g batches. Cells were disrupted in a Bead Beater (*31*) fitted with a dry ice/2-propanol cooling jacket for 6 × 2 min bursts with appropriate cooling intervals (1−2 min), and microsomal membranes were prepared as previously described (*30*). The yield of microsomes was ∼400 mg of total protein per 100 g of wet cells.

Solubilization and Purification of ABCG5 and ABCG8. Microsomes were thawed at room temperature and solubilized in 0.5% FosCholine-14 (FC-14, Anatrace) at a final concentration of 2 mg/mL in buffer A [50 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 30% glycerol] supplemented with 1 mM β-mercaptoethanol and protease inhibitors (10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 1 mM PMSF) for 10 min at 4 °C. The solubilized microsomes were centrifuged at 64000*g* for 30 min. The soluble supernatant was incubated overnight at 4 °C on a Labquake rotator with 4 mL of Ni^{2+}−NTA resin that was pre-equilibrated in buffer A. The resin was collected into a column and washed with 5 bed volumes of buffer A containing 0.1% FC-14. Proteins were eluted from the resin over a period of 30 min using 3 bed volumes of 200 mM imidazole buffer [50 mM Tris-HCl (pH 7.4), 20% glycerol, 50 mM NaCl, and 200 mM imidazole] containing 0.1% FC-14.

Four milliliters of calmodulin affinity resin was pre-equilibrated in $CaCl_2$ binding buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 20% glycerol]. The 200 mM imidazole eluate from the Ni−NTA column was diluted 17-fold with $CaCl_2$ binding buffer containing 0.1% FC-14 and incubated with the calmodulin resin for 4 h at 4 °C on a Labquake rotator. The resin was transferred into a column, and the flow-through fraction was collected. The resin was washed with 5 bed volumes of $CaCl_2$ binding buffer containing 0.1% FC-14. Proteins were eluted from the resin over a period of 60 min with 3 bed volumes of EGTA elution buffer [2 mM EGTA, 50 mM Tris-HCl (pH 7.4), 20% glycerol, and 150 mM NaCl] containing 0.1% FC-14. The protein was concentrated by ultrafiltration under

[1] Abbreviations: FC-14, FosCholine-14 or *n*-tetradecylphosphocholine; DDM, *n*-dodecyl β-D-maltopyranoside; lyso-PC, lysophosphatidylcholine; AlFx, fluoroaluminate; BeFx, beryllium fluoride; Vi, vanadate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(aminoethyl ether)tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; NBD, nucleotide binding domain; TM, transmembrane domain.

N_2 using an Amicon stirred cell with an XM-50 membrane (Millipore) to 1−2 mL, and the protein concentration was determined by UV spectroscopy. The concentrated material was aliquoted and stored at −80 °C. For a second extraction, the calmodulin resin was re-equilibrated with 10 bed volumes of $CaCl_2$ binding buffer and then re-incubated with the flow-through fraction from above overnight at 4 °C. The calmodulin resin was collected into a column, washed, and eluted, and the proteins were concentrated as described above.

Reconstitution of Purified ABCG5, ABCG8, and ABCG5/G8. Lipid stocks were prepared from *E. coli* lipids, liver lipids, liver PC lipids, egg PC lipids, or asolectin to a final concentration 20 mg of lipid/mL as described previously (*30*) and stored under N_2. For activation experiments, detergent-soluble protein was mixed with an equal volume of lipids and incubated at room temperature for 20 min and then sonicated in a bath sonicator at room temperature for 30 s. The activated protein was then stored on ice for a minimal period until the assay. For reconstitution experiments, ABCG5/G8 was mixed with an equal volume of *E. coli* lipids and incubated for 20 min at room temperature. Detergent was removed by (a) dialysis against 2 L of Tris buffer [50 mM Tris-HCl (pH 7.4)] at 4 °C with three buffer changes, each after 6−12 h, using dialysis tubing (Spectrapor) with a cutoff molecular mass of 10 000 kDa, (b) gel filtration chromatography on Sephadex G-50 resin (1 cm × 50 cm) in Tris buffer at 4 °C with a flow rate of 0.13 mL/min, or (c) rapid dilution into 50 volumes of ice-cold Tris buffer followed by centrifugation at 150000*g* at 4 °C to collect the proteoliposomes. Proteoliposomes were resuspended in Tris buffer and immediately assayed.

ATPase Assays. Reactions were carried out in 50 μL of 50 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$, 10 mM NaATP, and 1 mM sodium azide at 37 °C for appropriate times. Reactions were started by addition of 5−10 μg of ABCG5/G8 and stopped by the addition of 1 mL of 20 mM ice-cold H_2SO_4. Released P_i was assayed by the method of Van Veldhoven and Mannaerts (*32*). For the determination of kinetic parameters, MgATP concentrations were varied with an excess of 2 mM free Mg^{2+} over MgATP. Solutions of sterols were added in dimethyl sulfoxide, and the final concentration of the solvent was ≤2% (v/v) in the assays. Inhibition was assayed in cocktails containing 10 mM MgATP supplemented with either 0.2−1 mM orthovanadate, 0.2−1 mM $BeSO_4$ and 5 mM NaF (BeFx), or 1 mM $AlCl_3$ and 5 mM NaF (AlFx). Orthovanadate solutions (100 mM) were prepared from Na_3VO_4 (Fisher Scientific) at pH 10 and boiled for 2 min before each use to break down polymeric species (*33*).

Gel Filtration Chromatography. Protein (500 μg) was resolved on a Sephacryl-S300HR gel filtration column (1 cm × 100 cm) at 4 °C with a flow rate of 0.13 mL/min in buffers containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 0.01% FC-14. Protein elution was monitored with a UV detector (LP system, Bio-Rad), and 3 mL fractions were collected for analysis by SDS−PAGE. For this, proteins were absorbed to 10 μL of Strataclean resin (Stratagene) for 1 h on a Labquake rotator at 4 °C. The resin was centrifuged for 1 min at 14000*g* and 4 °C, and the supernatant was removed. Proteins were eluted from the resin by incubation with 10 μL of sample buffer and urea [125 mM Tris-HCl (pH 6.8), 5% SDS, 8 M urea, pyronin Y, and 160 mM DTT] at 85 °C for 5 min and centrifuged for 1 min, and the supernatant was resolved on 10% SDS gels and stained with Coomassie Brilliant Blue.

Routine Procedures. Concentrations of purified proteins were determined by UV spectroscopy using a calculated A_{280}-(1 mg/mL) of 0.7, 1.3, and 1.05 for ABCG5, ABCG8, and ABCG5/G8, respectively. SDS−PAGE was performed using a Mini-PROTEAN-3 gel and Electrotransfer system (Bio-Rad). Samples were dissolved in 0.5 volume of sample buffer [125 mM Tris-HCl (pH 6.8), 5% (w/v) SDS, 25% (v/v) glycerol, 0.01% pyronin Y, and 160 mM DTT] for 5 min at 85 °C and then resolved on 10% polyacrylamide gels. For immunodetection, monoclonal anti-RGS-His_4 antibody (Qiagen), polyclonal anti-ABCG5 antibody, or monoclonal anti-ABCG8 antibody was used with the TMB substrate system (Kirkegaard & Perry). LC−MS/MS analysis of tryptic digests (Sigma kit, catalog no. PP0100) was carried out on a Deca XP plus apparatus (Thermo Electron).

RESULTS

Cloning and Expression of the Human ABCG5 and ABCG8 Proteins. Previously, we utilized the yeast *P. pastoris* for expression and purification of fully functional P-glycoprotein (ABCB1), a full-size ABC transporter involved in multidrug resistance of cancer cells (*29*, *30*). Here, we have adopted this system for the expression and purification of the human ABCG5 and ABCG8 proteins. We first screened several transformants for the expression of ABCG5 and ABCG8 by Western blot analysis. Two of three ABCG5 transformants and three of three ABCG8 transformants exhibited a distinct protein band (∼70−80 kDa) that cross-reacted with an antibody against the C-terminal RGS-His_6 tag but was absent in the sham negative vector (Figure 1 of the Supporting Information).

Solubilization and Purification of ABCG5 and ABCG8. We selected the best expressers for growth in fermentor cultures and isolated the microsomal membrane fraction to collect proteins present in plasma membranes and in intracellular membranes (*30*). Next, we conducted solubility experiments in a variety of detergents to extract the proteins from the membranes under mild conditions. We selected detergents which we previously used for the purification of highly active P-glycoprotein (*29*, *30*), as well as synthetic lyso-PC derivatives with defined hydrocarbon chains (e.g., FC-14 has a C14 chain), and other detergents that are routinely used in the purification of membrane proteins (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Nine detergents (DM, DDM, lyso-PC, LDAO, Cyfos5, Cyfos6, and FosCholine-12, -14, and -16) were efficient and were selected for small-scale purification trials on Ni−NTA resin. For both ABCG5 and ABCG8, the highest yields were obtained using the detergent FC-14 (data not shown) which was subsequently chosen for large-scale purifications.

The procedure we developed for the purification of ABCG5 and ABCG8 is described in detail in Experimental Procedures and is based on methods we have successfully applied to the purification of P-glycoprotein from *P. pastoris* membranes (*30*). Briefly, the proteins were purified by tandem affinity chromatography on Ni−NTA and calmodulin affinity resin after solubilization of microsomal membranes

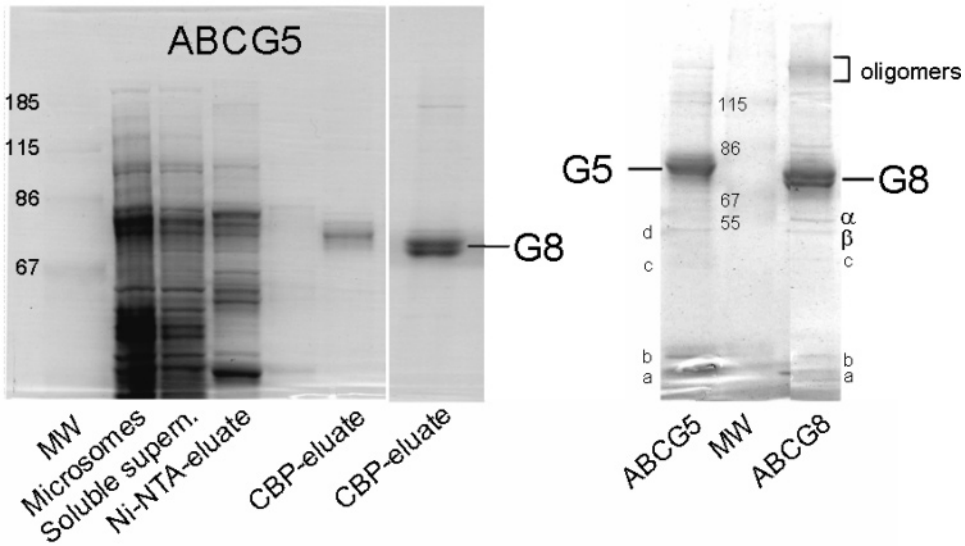

FIGURE 1: Purification of ABCG8 and ABCG5. The progress of the purification for ABCG5 in FC-14 is shown after consecutive chromatography steps on Ni−NTA and calmodulin (CBP) resins. Protein samples were resolved via SDS−PAGE followed by Coomassie blue staining. ABCG8 was purified following the same procedure, and the final pure protein is shown. Ten micrograms each of concentrated ABCG5 and ABCG8 are shown in the right panel. The positions of the molecular mass protein markers are given in kilodaltons. α and β indicate the position of the α- and β-subunits of F_1-ATPase, respectively; a−d are degradation products of ABCG5 and ABCG8.

Table 1: Purification Yields of ABCG5, ABCG8, and ABCG5/G8

strain	detergent	yield[a]	no. of purifications
ABCG5	FC-14	0.74	3
ABCG8	FC-14	1.50	4
ABCG5/G8			
transformant 1	FC-14	8.7	3
transformant 2	FC-14	5.5	2
ABCG5/G8	DDM	2.1	2

[a] The yield is expressed as milligrams of protein per 100 g of cells. Copurification of ABCG5/G8 resulted in up to 11.8- and 5.8-fold higher yields than purification of ABCG5 and ABCG8 alone, respectively, in the same detergent (FC-14). The highest yields were consistently obtained in FC-14.

Table 2: ATPase Activity of ABCG5, ABCG8, and ABCG5/G8

protein	ATPase activity[a] (nmol min^{-1} mg^{-1})	K_M (mM)	Hill coefficient	k_{cat}/K_M (M^{-1} s^{-1})
ABCG5	4 ± 3	ND[b]	−	−
ABCG8	3 ± 2[c]	ND[b]	−	−
ABCG5/G8[d]	15 ± 5	4 ± 2[e]	1.7 ± 0.3[e]	11

[a] Purified, detergent-soluble proteins were activated with liver lipids, and ATPase activity was assayed in cocktails containing 10 mM MgATP and 1 mM sodium azide. The averages of standard deviations of at least 20 independent experiments are given. [b] Not determined because of low activity. [c] Contamination by F_1-ATPase activity was detected in three of four ABCG8 purifications by Western blot analysis. Shown is the ATPase activity of the ABCG8 preparation that was free of F_1-ATPase. [d] ABCG5 and ABCG8 are individually purified proteins, and ABCG5/G8 represents coexpressed and copurified proteins. [e] Averages and standard deviations of six independent experiments.

in FC-14. The progress of the purification of ABCG5 and the final pure ABCG5 and ABCG8 were visualized on a Coomassie Blue-stained SDS gel (Figure 1). The yields were approximately 0.7 and 1.5 mg for ABCG5 and ABCG8, respectively, from 100 g of yeast cells in three to four independent purifications of each protein (Table 1). The final pure materials were in buffers with low concentrations (0.1%, w/v) of FC-14 and behaved as soluble proteins.

ATPase Activity of the Individual ABC Transporters. ABCG5 and ABCG8 belong to the family of ABC transporters and are expected to hydrolyze ATP to drive transport. We therefore examined whether the individually purified transporters are active enzymes capable of hydrolyzing ATP. Purified, detergent-soluble ABCG5 and ABCG8 were activated with liver lipids or *E. coli* lipids (see Experimental Procedures), and their ATPase activities were assayed over a period of up to 4 h at 37 °C. Pure ABCG5 from three independent purifications had very low but detectable ATPase activity of 4 ± 3 nmol min^{-1} mg^{-1} (Table 2).

The level of ATP hydrolysis by ABCG8 was surprisingly high and varied significantly between preparations, reaching as high as 500 nmol min^{-1} mg^{-1} and as low as 3 ± 2 nmol min^{-1} mg^{-1}. Therefore, we suspected contamination by other ATPases in some of the preparations. Contamination by the most prominent plasma membrane H^+-ATPase (PMA1) in yeast microsomes (*34*) could be ruled out since the activity was not sensitive to vanadate, a potent inhibitor of P-type ATPases. To find out which other ATPases may have copurified with ABCG8, we loaded large amounts of purified proteins on SDS gels and subjected all bands that could be detected by Coomassie blue staining to LC−MS/MS analysis. Two bands barely visible in Figure 1 (right panel) were identified as the α- and β-subunits of F_1-ATPase using the database of *Saccharomyces cerevisiae* (this yeast is similar to *P. pastoris*). Western blot analysis using specific antibodies confirmed the presence of the β-subunit of F_1-ATPase in three of four ABCG8 preparations. However, this ATPase was completely absent in all ABCG5 preparations (Figure 2 of the Supporting Information). Sodium azide, a known inhibitor of F_1-ATPase, inhibited the three contaminated ABCG8 preparations in a concentration-dependent manner with >95% inhibition seen at concentrations of ≥1 mM. As a precaution, we included 1 mM sodium azide in all subsequent ATPase assays. The ATPase activity of the one ABCG8 preparation free of F_1-ATPase, as determined by Western blot analysis, was not sensitive to sodium azide and had an ATPase activity of 3 ± 2 nmol min^{-1} mg^{-1} (Table 2). Thus, we concluded that the ATPase activity of ABCG8

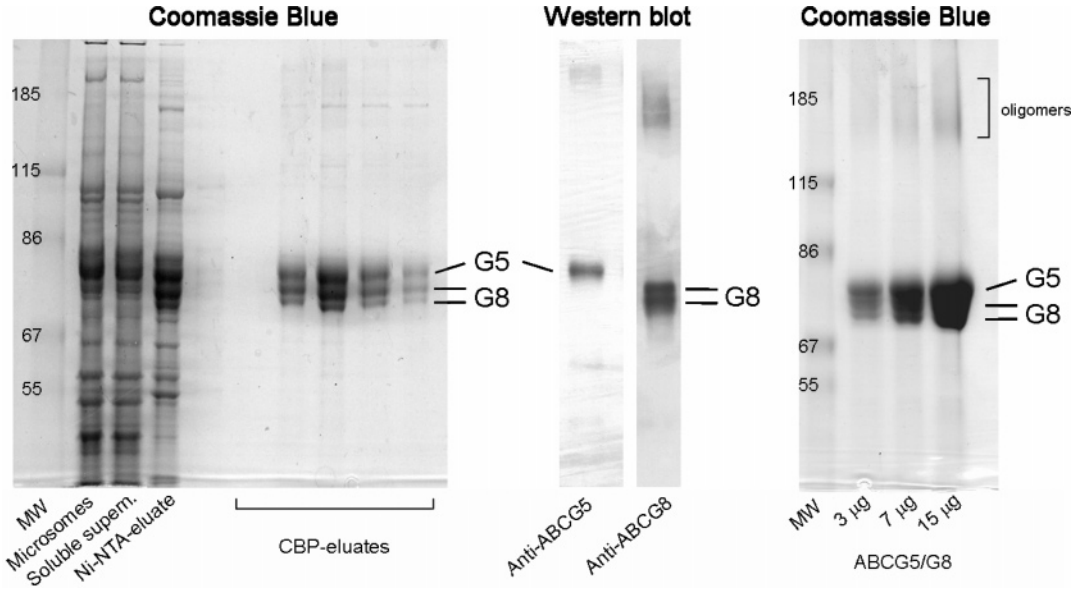

FIGURE 2: Purification of ABCG5/ABCG8. The progress of the purification for ABCG5/G8 in FC-14 is shown. Protein samples were resolved via SDS−PAGE followed by Coomassie blue staining (left panel). Ten microliters of each 1 mL fraction eluted from the calmodulin resin (CBP eluates) was analyzed. The identity of the ABCG5 and ABCG8 proteins was confirmed by Western blotting using a polyclonal anti-ABCG5 and a monoclonal anti-ABCG8 antibody (middle panel). Purified, concentrated ABCG5/G8 is shown in the right panel. The positions of the molecular mass protein markers are given in kilodaltons.

is also very low (similar to ABCG5) and may even be close to zero since we cannot rule out a contribution by other low-level ATPases that may not have been detectable by MS analysis.

*Coexpression and Copurification of ABCG5/G8***.** ABCG5 and ABCG8 have been suggested to function as heterodimers (see the introductory section); therefore, we tried to coexpress the two genes together in *P. pastoris*. In this yeast, the transformed genes are integrated into the genome, and integration of multiple copies of the plasmids is associated with an increasing level of resistance to Zeocin. Our approach was to cotransform the two plasmids containing the individual ORFs and to select for multicopy integrations by plating on media containing increasing concentrations of Zeocin. Twelve colonies were obtained on plates containing 500 μg/mL Zeocin and screened by rapid membrane preparation and Western blot analysis. Of these, four coexpressed both ABCG5 and ABCG8 as determined by Western blotting with a polyclonal anti-ABCG5 and a monoclonal anti-ABCG8 antibody (Figure 2 of the Supporting Information). Furthermore, the expression levels of two of the coexpressers (termed ABCG5/G8) were significantly increased (5−10-fold) compared to those of transformants expressing the single genes (data not shown).

We have grown the two best ABCG5/G8 coexpressers in fermentor cultures and purified the proteins following the same procedures as described for the single genes. It was immediately apparent on Coomassie blue-stained gels that the proteins eluted with much higher yields from the Ni−NTA and calmodulin resins (Figure 2) than when they were purified individually. The concentration of ABCG5/G8 reached 0.5 mg/mL in the final CBP eluates, and ABCG5 and ABCG8 coeluted in the same fractions. We then loaded larger amounts of concentrated ABCG5/G8 on SDS gels (up to 15 μg) and saw very few contaminants. Of these, minor bands visible at >150 kDa were oligomeric proteins as demonstrated by Western blotting. The total yield of the concentrated pure protein ranged between 5 and 9 mg of pure protein per 100 g of yeast cells, dependent on the particular transformant that was used (Table 1). The 5−10-fold higher yields of coexpressed ABCG5/G8, compared to those of the individually expressed proteins (Table 1), support the idea that the two transporters tightly interact and stabilize each other during expression and purification.

Purified proteins from the coexpressing strains were separated into three distinct bands when resolved on 7.5% SDS gels (Figure 2). Western blot analysis using specific antibodies revealed that ABCG5 resided in the uppermost band while ABCG8 split into the two lower bands (Figure 2, middle panel). This was confirmed by N-terminal sequencing of the individual proteins, which revealed that both proteins started with their predicted methionine at position 1 and that proteolytic degradation at the N-terminus was not the cause of the differences seen in sizes. ABCG5 has two N-linked glycosylation sites (N585 and N592), while ABCG8 has a single site (N619) that can be glycosylated (*27*). To address the glycosylation patterns of ABCG8 and ABCG5/G8 after expression in *P. pastoris*, we treated the pure proteins with endoglycosidase EndoH, which removes N-linked glycans of the mannose type typically seen in *P. pastoris* proteins (*35*). Treatment with EndoH resulted in the collapse of the doublet and triplet of ABCG8 and ABCG5/G8, respectively, into a single protein band (Figure 3). The size of this band corresponds to ~75 kDa monomeric proteins. We thus concluded that ABCG5 in our preparations is consistently glycosylated at two sites and that ABCG8 is either glycosylated at one site or not glycosylated, resulting in the doublet band. Proteins expressed in *P. pastoris* are usually core-glycosylated with glycans of the mannose type (*36*), which is distinct from the extensive glycosylation pattern of ABCG5 and ABCG8 seen in mammalian cells (*14*). This provides an explanation for the relatively small differences in size seen after treatment with EndoH and for the apparent lower molecular masses seen for ABCG5 and

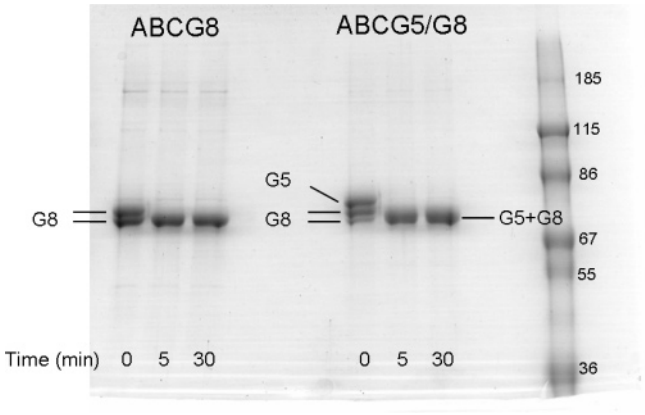

FIGURE 3: EndoH treatment of ABCG8 and ABCG5/G8. Purified ABCG8 or ABCG5/ABCG8 was treated with 500 units of EndoH (NEB) at 37 °C for the times indicated, and the proteins were resolved on 10% SDS gels and stained with Coomassie blue. Similar results were seen for ABCG5 (not shown). Sizes of the molecular mass markers (kilodaltons) are given at the right.

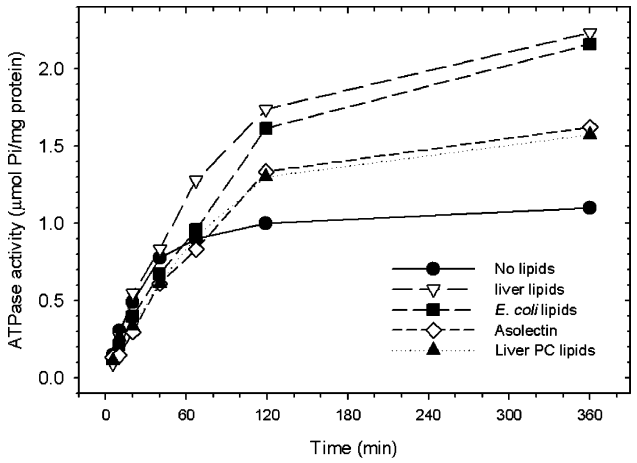

FIGURE 4: ATPase activity of ABCG5/G8. Purified, detergent-soluble ABCG5/G8 was either directly diluted into 10 mM MgATP cocktail or activated with 1% lipids at room temperature for 20 min followed by a 30 s sonication prior to dilution into ATP cocktail. ATP hydrolysis was assayed for the indicated times at 37 °C and the amount of inorganic phosphate determined as described in Experimental Procedures. Average values from 10−20 independent data points are shown. Standard deviations were small and thus omitted for clarity. Similar results were seen with egg PC lipids as well as mixtures of purified phospholipids that resemble the plasma membrane composition more closely (*42*).

ABCG8 expressed in *P. pastoris* compared to the proteins in mammalian cells.

ATPase Activity of Copurified ABCG5/G8. To analyze the ATPase activity of coexpressed heteromeric ABCG5/G8, the detergent-soluble protein was diluted into cocktails containing 10 mM MgATP and assayed over a period of 6 h. ATP hydrolysis was readily apparent, and the level of hydrolysis was found to increase over time [Figure 4(●)]. However, a decrease in activity was observed with longer incubations (>40 min), indicating that the proteins in detergent-soluble form are somewhat unstable at 37 °C.

We then decided to explore whether reconstitutions of the purified proteins into lipids from different sources and lipid composition stabilize the transporters and/or increase their ATPase activities. In this analysis, we included extracts from liver lipids (containing PC and cholesterol), *E. coli* lipids (devoid of PC and cholesterol), and asolectin (containing plant sterols). We employed an "activation" procedure previously demonstrated to give maximum ATPase activity of P-glycoprotein without lengthy removal of the remaining detergent (*30*). In the presence of lipids, the rate of ATP hydrolysis increased linearly over ~60 min and slowed thereafter. The initial rates were similar under all the conditions that were tested, and the only difference we observed was an increase in the stability of the enzyme in different lipid environments over time (Figure 4). Specific ATPase activities of the initial time points ranged from 10 to 20 nmol min^{-1} mg^{-1} in >20 independent experiments (K_{cat} ~ 2.5 ATP molecules hydrolyzed/min). This provides the first direct evidence that the heteromer is an active form of the transporters. Activation of ABCG5/G8 with liver lipids or *E. coli* lipids resulted in the most prolonged and highest activities (Figure 4) which implies that these lipids stabilize the purified ABCG5/G8 proteins.

Effects of Sterols on the ABCG5/G8 ATPase. Binding of substrate to the TMs of ABC transporters typically stimulates hydrolysis at the NBDs to energize the transport process. Therefore, we tested the effects of the predicted transport substrates of ABCG5/G8, cholesterol, and the plant sterols sitosterol and stigmasterol on the ATPase activity of ABCG5/G8. ABCG5/G8, either in a detergent solution or activated with *E. coli* lipids (which do not contain cholesterol), were assayed in the presence of increasing amounts of sterols over a wide range of concentrations from 10 nM to 100 μM. However, no stimulation of ATPase was observed as the specific activities were similar in the absence or presence of sterols [~15 ± 5 nmol min^{-1} mg^{-1} (data not shown)].

To investigate whether the detergent FC-14 present in the preparation interferes with the binding of sterols to the potential binding sites within the TM regions, we reconstituted ABCG5/G8 into *E. coli* proteoliposomes (see Experimental Procedures). Reconstitution was conducted via either gel filtration chromatography, rapid dilution, or dialysis in repeated experiments. However, the results from different reconstitution procedures were similar and were not affected by the presence or absence of residual detergent. Furthermore, we purified ABCG5/G8 in the detergent DDM to examine the possibility that the lack of stimulation was associated with the detergent FC-14 in particular. The purity of ABCG5/G8 in DDM was comparable to that of proteins purified in FC-14, although the yield was lower (2.1 mg/100 g of cells, Table 1). The specific activity of the DDM-purified proteins was 18 ± 3 nmol min^{-1} mg^{-1} (Table 2). No stimulation of this activity by sterols was observed. It is noteworthy that P-glycoprotein, which we routinely purify in DDM, displays significant stimulation of ATPase activity (5−13-fold) by drugs under the same assay conditions that were applied here (*31*). Thus, on the basis of the result of our extensive analysis, detergents per se are not likely to be a primary cause of interference, and we conclude that sterols do not stimulate the ATP hydrolysis of purified heteromeric ABCG5/G8.

Determination of the Oligomeric Status of the Purified Proteins. To determine the oligomeric status of the purified proteins, we utilized size exclusion chromatography on Sephacryl S-300. A typical elution profile for ABCG8 is shown in Figure 5 (solid line), with the molecular mass markers indicated at the top. ABCG8 eluted as a sharp peak

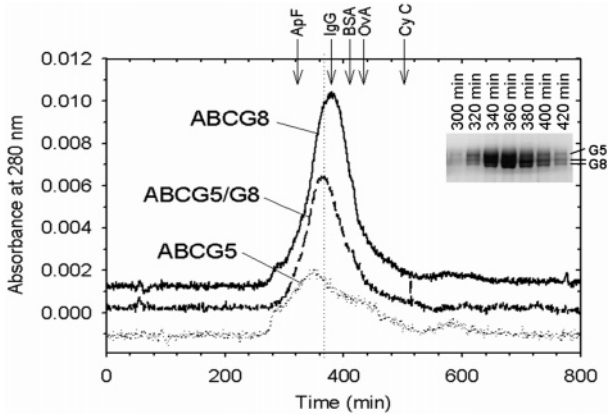

FIGURE 5: Size exclusion chromatography of ABCG5, ABCG8, and ABCG5/G8. Five hundred micrograms of purified, detergent-soluble proteins was loaded on a Sephacryl S-300 HR column and resolved in buffers containing small amounts of detergent (Experimental Procedures). A representative of three independent runs is shown for ABCG8 (—), ABCG5 (···), and coexpressed ABCG5/G8 (– – –). The A_{280} for ABCG5 was approximately half the absorbance of ABCG8. Molecular mass markers were resolved under identical buffer conditions, and the retention times are given at the top: ApF (apoferritin), 380 kDa; IgG, 150 kDa; BSA, 65 kDa; OvA (ovalbumin), 45 kDa; and CyC (cytochrome *c*), 13 kDa. The calculated molecular masses of monomeric ABCG5 and ABCG8 (including the affinity purification tags) and of dimeric ABCG5/G8 are 79, 82, and 161 kDa, respectively. The predicted detergent/micelle size for FC-14 is ~20 kDa. In the inset, after chromatography of ABCG5/G8, 3 mL fractions were collected, precipitated, resolved on SDS gels, and stained with Coomassie blue.

with a retention time of 375 min, identical to that of IgG; thus, the purified form of ABCG8 appears to be a homodimer of ~160 kDa. The data suggest that ABCG8, in the absence of ABCG5, homo-dimerized to form a somewhat stable complex. Conversely, ABCG5 eluted over a wide range between 320 and 440 min, suggesting a mixture of higher oligomeric and monomeric proteins in the purified protein preparation (Figure 5, dotted line). The abnormal elution pattern of ABCG5, together with the low yield of the purified protein (Table 1), suggests that this transporter is somewhat unstable in solution.

ABCG5/G8 eluted with a retention time of ~355−360 min (Figure 5, dashed line), slightly earlier than IgG (and ABCG8). This suggests that the majority of the protein likely exists as a dimer. However, because of the slight left shift of the curve compared to ABCG8 and a rather broad shape of the peak, we cannot exclude the possibility that some of the proteins exist as higher oligomeric species. Analysis of the protein peak by SDS−PAGE revealed approximately equimolar amounts of ABCG5 and ABCG8 in all fractions of the peak (Figure 5, inset). Collectively, this later result, the left shift of ABCG5/G8 compared to ABCG8, and the distinctly different profile of ABCG5 argue for a heterodimeric species in our ABCG5/G8 preparation.

Parameters of ATP Hydrolysis of ABCG5/G8. Kinetic parameters of ATP hydrolysis were determined from multiple experiments after activation of the purified proteins with liver lipids (Table 2). We observed maximal ATP hydrolysis by ABCG5/G8 at concentrations of ≥10 mM MgATP with a V_{max} of ~16 nmol min^{-1} mg^{-1} (k_{cat} = 2.6 min^{-1}). K_M-(MgATP) was relatively high (~4 mM, Figure 6). The best fit of the data was obtained using the Hill equation (Figure 6, solid lines) with a Hill coefficient of 1.7. This finding indicates a strong cooperativity between the two NBDs for ATP hydrolysis. In addition, MgADP behaved as a competitive inhibitor of MgATP hydrolysis with a relatively high K_i of 0.4 mM.

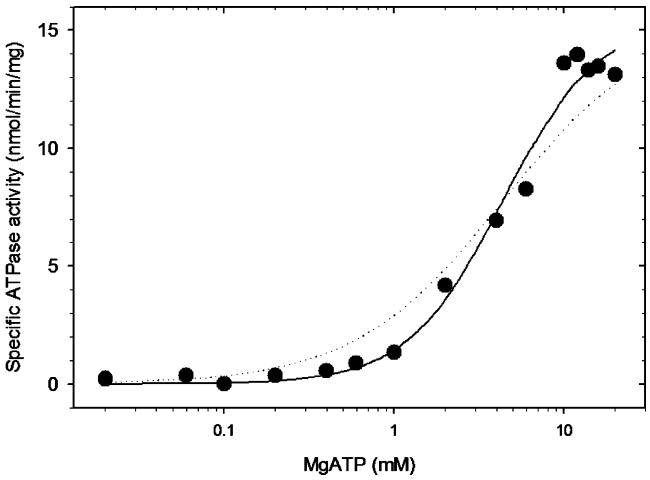

FIGURE 6: MgATP hydrolysis of ABCG5/G8. Purified ABCG5/G8 was activated with liver lipids and assayed for 3 h at 37 °C with varying concentrations of MgATP: (—) fit to the Hill equation with a Hill coefficient of 1.8 and (···) fit to the Michaelis−Menten equation expected for simple, noncooperative binding. The figure shows a representative result from six independent experiments.

In the absence of sodium azide, the ATPase activity was 50 ± 30 nmol min^{-1} mg^{-1}. Therefore, we analyzed all our ABCG5/G8 preparations for the presence of F_1-ATPase. Only when we loaded 10 μg of purified proteins could we detect this ATPase, suggesting a very low level of contamination (Figure 3 of the Supporting Information). Concentrations of 1 mM sodium azide were sufficient to inhibit this contaminating ATPase since no further inhibition was observed at 10 or 20 mM sodium azide. Divalent metal ions were essential cofactors for ATP hydrolysis since no activity was detected in their absence (and in the presence of 5 mM EDTA). Co^{2+}, Mn^{2+}, and Ca^{2+} all supported ATP hydrolysis of ABCG5/G8, although the relative ATPase activity was lower than with Mg^{2+}, resulting in ~51, ~43, or ~18% of the MgATP hydrolysis rate, respectively, when assayed at 10 mM Me^{2+}ATP. The Mg nucleoside triphosphates CTP, GTP, ITP, and UTP were hydrolyzed at less than 5% of the rate for MgATP, indicating a high specificity for ATP. Assessment of MgATP hydrolysis at varying pH values revealed a broad pH optimum between 7 and 9.0 (Figure 7). ATPase activities were very similar when assayed in MgATP cocktails containing 50 mM Tris-HCl, Hepes, MOPS, or TES buffer at pH 7.4. Preincubation of ABCG5/G8 with 10 mM DTT had no effect on the ATPase activity, indicating that cysteines are not critical for ATP hydrolysis. Finally, pretreatment of ABCG5/G8 with EndoH did not change the ATPase activity. Thus, removal of glycans as seen in Figure 3 did not affect the activity of the transporters.

Inhibition of the ABCG5/G8 ATPase by Transition- and Ground-State Analogues. Transition- and ground-state analogues have been utilized extensively in examining the catalytic mechanism of P-glycoprotein and other ABC transporters. We tested these analogues for their ability to inhibit the ABCG5/G8 ATPase. AlFx and BeFx were both effective and nearly abolished the ATP hydrolysis at

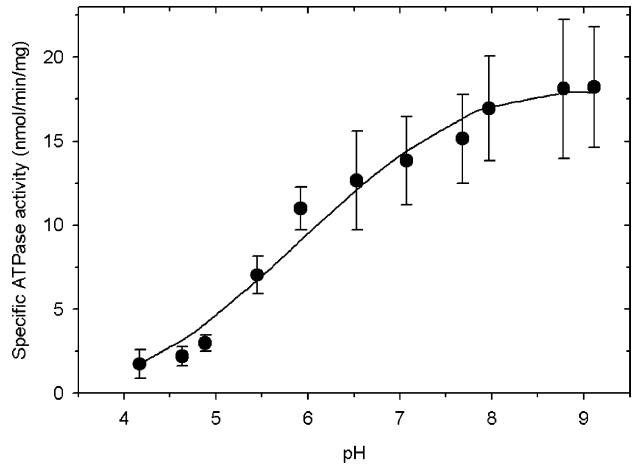

FIGURE 7: pH dependence of the ATPase activity of purified ABCG5/G8. The ATPase activity was measured in 50 mM Tris-succinate buffers as described previously (*53*). Results are averages of duplicate determinations from two independent experiments ± standard deviations (bars).

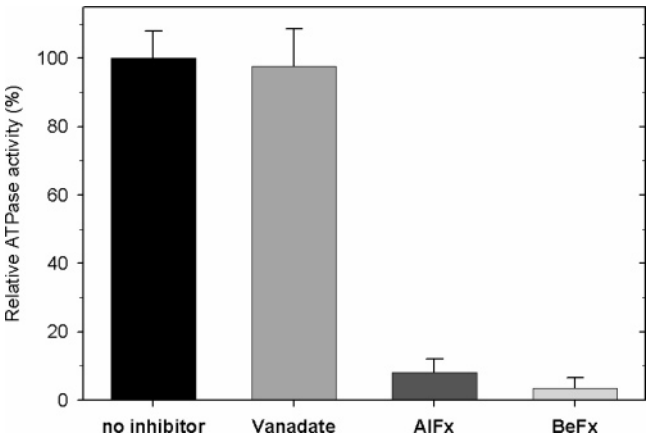

FIGURE 8: Inhibition of ATPase activity of ABCG5/G8 by transition-state analogues. Purified ABCG5/G8 was activated with liver lipids and ATPase activity assayed for 30 min at 37 °C. The reaction cocktails contained 10 mM MgATP and inhibitors at the following concentrations: 200 μM vanadate, 200 μM $BeSO_4$ with 1 mM NaF (BeFx), and 1 mM $AlCl_3$ with 5 mM NaF (AlFx). One hundred percent activity (in the absence of inhibitor) was 20 ± 3 nmol min^{-1} mg^{-1}. The data are means of at least five independent experiments.

concentrations of 200 μM and 1 mM, respectively (Figure 8). However, ABCG5/G8 exhibited little inhibition by orthovanadate, retaining 98% of the activity at 200 μM vanadate and 89% activity at 1 mM vanadate. The data suggest that the coordination around the γ-phosphate during hydrolysis of ATP, as mimicked by vanadate in the transition-state complex, is somewhat different in ABCG5/G8 compared to P-glycoprotein (*37*).

Demonstration that BeFx- and AlFx-Induced Inhibition Involves Trapping of Nucleotide. The autoradiograph in Figure 9A shows that inhibition by BeFx was associated with trapping of 8-azido[α-^{32}P]nucleotides in the ABCG5/G8 protein bands. Trapping was specific to ABCG5/G8, and the trapped nucleotide was not displaced by excess cold ATP. The presence of liver lipids enhanced trapping (Figure 9, lane 1 vs lane 2), suggesting that these lipids affect the conformation of the transporters. Trapping has been shown to require hydrolysis of the nucleotide triphosphates in other ABC transporters (*38*), and therefore, the data provide

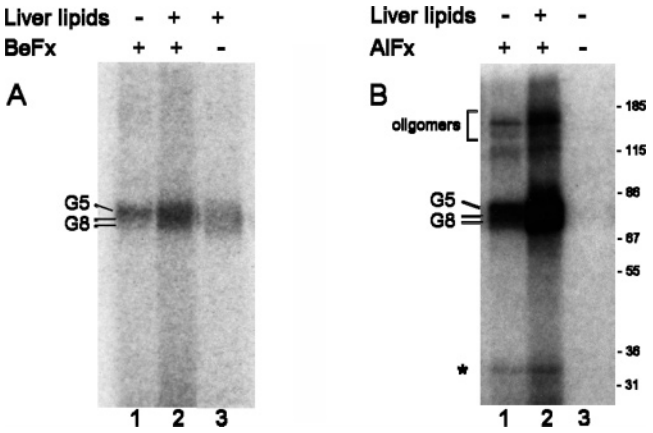

FIGURE 9: Nucleotide trapping in ABCG5/G8. Purified ABCG5/G8 (3.5 μg) either in a detergent solution or activated with liver lipids as indicated at the top of the lanes were reacted with 50 μM 8-azido[α-^{32}P]ATP, 4 mM $MgCl_2$, 1 mM sodium azide, in the absence or presence of (A) 500 μM $BeSO_4$ and 5 mM NaF (BeFx) or (B) 1 mM $AlCl_3$ and 5 mM NaF (AlFx) for 15 min at 37 °C. Excess 30 mM MgATP was added to displace loosely bound radionucleotides, and the proteins were UV irradiated for 7 min on ice. Proteins were resolved on SDS gels and exposed to Biomax MS film. The low level of radionucleotide seen in lane 3 of panel A is presumably due to incomplete displacement of nucleotide under these experimental conditions. The asterisk denotes degradation products of ABCG5/G8.

convincing evidence that ABCG5/G8 is indeed capable of hydrolyzing ATP. Furthermore, no BeFx-dependent trapping of 8-azido nucleotide was observed in purified ABCG5 or ABCG8 (not shown), suggesting that these homomers do not hydrolyze 8-azido-ATP efficiently enough to produce trapping of the nucleotide.

We observed qualitatively similar results when AlFx was used (Figure 9B). Moreover, the intensity of the signal for the trapped nucleotide in the ABCG5/G8 bands significantly increased with this transition-state analogue compared to the ground-state analogue BeFx (Figure 9A). Liver lipids again stimulated hydrolysis and trapping (lane 1 vs lane 2), but no radioactivity was found in the ABCG5/G8 bands in the absence of AlFx. Notably, no radioactivity was observed at the position of the catalytic β-subunit of F_1-ATPase (~55 kDa). This is important because it shows that, under the experimental conditions used here, hydrolysis and trapping were specific to ABCG5/G8.

DISCUSSION

In this work, we purified for the first time the putative cholesterol transporters ABCG5 and ABCG8 after expression in the yeast *P. pastoris*. Both proteins were >85% pure (Figure 1) and, as prepared, were soluble in 0.1% FC-14 and remained soluble at much lower concentrations of the detergent. We also show that coexpression of the two ABC transporters, after simple cotransformation of the two genes into *P. pastoris*, significantly increased both the yield and purity of the ABCG5/G8 proteins (Figure 2 and Table 1). A typical fermentor culture yields ~1.5 kg of cells, which will potentially yield 130 mg of copurified ABCG5/G8. The availability of large amounts of pure and active proteins has important implications for future biochemical, biophysical, and structural studies. Therefore, our work represents the first step toward future structure−function studies that will potentially lead to elucidation of the mechanism(s) by which

these suggested heterodimeric transporters contribute to arteriosclerosis.

As ABC transporters, ABCG5 and ABCG8 are expected to hydrolyze ATP to drive transport; therefore, we measured the ATPase activities of the individual and coexpressed transporters to reveal potential differences in their function. Individually expressed proteins had very low ATPase activities (Table 1), indicating that ABCG5 and ABCG8 by themselves may not be ATP-driven pumps. In contrast, coexpressed and copurified ABCG5/G8 displayed low but significant ATPase activity ($\sim$15 nmol min^{-1} mg^{-1}), which was stabilized by lipids from different sources (Figure 4). ABCG5, unlike ABCG8, did not elute in a clear dimer peak on gel filtration columns (Figure 5), and thus, we cannot exclude the possibility that expression of ABCG5 in yeast and subsequent purification yielded an inactive protein. However, in the coexpressed and copurified ABCG5/G8 preparations, ABCG5 obviously interacted with ABCG8 as suggested by the coelution of the two proteins during gel filtration chromatography and by the measurable ATPase activity, which also correlated with 8-azido nucleotide trapping. All these data correlate with the in vivo findings (see the introductory section).

The low ATP hydrolysis rates pose an experimental challenge for the determination of the ABCG5/G8 specific activity even of highly purified material because the slightest contamination by other ATPase may obscure the results. However, the kinetic parameters of ATP hydrolysis and the inhibitor profiles of ABCG5/G8 are distinctly different from those of other ATPases found in yeast membranes. First, the ABCG5/G8 catalytic sites displayed relatively low affinities for MgATP ($K_m \sim 4$ mM). This K_m is more than 10-fold higher than that reported for F_1-ATPase and most other ATPases in yeast (*39*−*41*) but is in a range typical for ABC transporters (*36*, *42*). Second, the phosphate analogues BeFx and AlFx, but not orthovanadate, inhibited MgATP hydrolysis. Finally, inhibition by BeFx and AlFx was concurrent with trapping of nucleotide in the ABCG5/G8 proteins. Trapping has been demonstrated to require hydrolysis of ATP or 8-azido-ATP in other ABC transporters (*38*, *43*). Therefore, specific labeling of the ABCG5/G8 bands in our experiments (Figure 9) provides convincing evidence that the ATP hydrolysis seen in the purified material is indeed due to these ABC transporters and not to contaminating proteins.

In ABC transporters, binding of the substrate to the TMs typically stimulates ATP hydrolysis at the NBDs, and the energy from the hydrolysis is then used to drive transport across membranes (*24*). Monitoring the ATPase activity in the presence of substrates has therefore been an important tool in assessing the substrate specificity of numerous ABC transporters and would be especially valuable here because transport assays with substrates as hydrophobic as cholesterol are difficult and have not been developed (*17*, *44*). However, the ATPase activity of purified and reconstituted ABCG5/G8 was not stimulated by the suggested transport substrate cholesterol and the plant sterols sitosterol and stigmasterol. The reason for this observation is not clear and certainly needs further investigation.

The observed rates of ATP hydrolysis for ABCG5/G8 are $\sim$100-fold lower than those of human P-glycoprotein (1700 nmol min^{-1} mg^{-1}) (*36*); however, they are similar to those reported for some other purified human ABC transporters such as CFTR, MRP1, and ABCR (1.7, 10, and 27 nmol min^{-1} mg^{-1}, respectively) (*45*−*47*). A possible explanation for the low ATPase activity seen in ABCG5/G8 as well as in CFTR, MRP1, and ABCR is that they all contain nonconserved amino acid residues within the conserved motifs essential for ATP hydrolysis (*48*). ABCG8 contains two nonconserved residues in the Walker A motif (GSSGCG**RA**S), and ABCG5 contains two nonconserved residues in the "signature" sequence (**I**S**T**GE) (*1*). X-ray structures of bacterial transporters show that as the two NBDs dimerize, they form two distinct sites for ATP hydrolysis at the dimer interface (*49*, *50*). In each site, the key residues involved in the interactions with ATP are derived from the highly conserved Walker A and B motifs on one NBD and from the so-called signature motif on the other NBD. Mutational alteration of either of the two conserved Walker A residues in P-glycoprotein (which contains two active sites) completely abrogates ATP hydrolysis in both sites (*29*, *51*). Substitutions of conserved residues in the signature sequences of P-glycoprotein also impair function, but to a lesser extent since substitutions in both NBDs are necessary to abolish the transporter's function (*52*). By analogy to these studies on P-glycoprotein, it seems likely that the very low ATP hydrolysis rates in separately purified ABCG5 and ABCG8 (Table 1) reflect the fact that each ATP-binding site in homodimers contains one nonconserved motif. In contrast, the ABCG5/G8 heterodimer may contain one potentially "active" site (two conserved motifs) and one degenerate site (two nonconserved motifs), strongly suggesting that this species is an active form of the transporter. However, because of the severe effects seen for the P-glycoprotein Walker A mutants, we would predict that the presence of even one noncanonical Walker A site in the ABCG5/G8 heterodimer is sufficient to render this a slow ATP-hydrolyzing enzyme. Our data confirm such a prediction.

A recent study by Zhang et al. has already shed light on the functional asymmetry of the NBDs in ABCG5 and ABCG8 in membrane preparations of mammalian cultures (*44*). The group found that (i) significant trapping of nucleotide occurs in the coexpressed heteromeric ABCG5/G8 transporters with both AlFx and BeFx under hydrolysis conditions, e.g., with 8-azido-ATP; (ii) trapping is also apparent in the individually expressed ABCG5, although to a lower extent; and (iii) trapping is completely absent in the ABCG8 homodimer, thus indicating that this NBD does not hydrolyze ATP. Furthermore, they also showed that mutations in the active site of the transporter pair severely compromise biliary sterol transport in transgenic mouse models, while mutations in the degenerate site had little effect on function. These recent data further support the prediction that the heterodimer is the active form of the transporters in vivo. Our findings are thus consistent with the results obtained from mammalian cultures and transgenic mouse models. Moreover, they validate our approach to purifying ABCG5/G8 from yeast to produce sufficient quantities of active proteins for further biochemical studies.

ACKNOWLEDGMENT

We are grateful to A. E. Senior for helpful discussion and Delphine Jean, Shiloe Souza, Taylor Strong, Mandy A. Geryk, and Ganesh Shankarling for excellent technical

assistance. We also thank W. S. Brusilow for anti-F_1-ATPase, Novus Biologicals for anti-ABCG5, and Helen Hobbs for anti-ABCG8 antibodies.

SUPPORTING INFORMATION AVAILABLE

Expression analysis of ABCG5 and ABCG8, coexpression analysis of ABCG5/G8, and analysis of copurification of F_1-ATPase in different preparations by Western blotting are shown in Figures 1−3 of the Supporting Information, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Berge, K. E., Tian, H., Graf, G. A., Yu, L., Grishin, N. V., Schultz, J., Kwiterovich, P., Shan, B., Barnes, R., and Hobbs, H. H. (2000) Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters, *Science 290*, 1771−1775.
2. Lee, M. H., Lu, K., Hazard, S., Yu, H., Shulenin, S., Hidaka, H., Kojima, H., Allikmets, R., Sakuma, N., Pegoraro, R., Srivastava, A. K., Salen, G., Dean, M., and Patel, S. B. (2001) Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption, *Nat. Genet. 27*, 79−83.
3. Lu, K., Lee, M. H., Hazard, S., Brooks-Wilson, A., Hidaka, H., Kojima, H., Ose, L., Stalenhoef, A. F., Mietinnen, T., Bjorkhem, I., Bruckert, E., Pandya, A., Brewer, H. B., Jr., Salen, G., Dean, M., Srivastava, A., and Patel, S. B. (2001) Two genes that map to the STSL locus cause sitosterolemia: Genomic structure and spectrum of mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively, *Am. J. Hum. Genet. 69*, 278−290.
4. Bhattacharyya, A. K., and Connor, W. E. (1974) β-Sitosterolemia and xanthomatosis. A newly described lipid storage disease in two sisters, *J. Clin. Invest. 53*, 1033−1043.
5. Miettinen, T. A. (1980) Phytosterolaemia, xanthomatosis and premature atherosclerotic arterial disease: A case with high plant sterol absorption, impaired sterol elimination and low cholesterol synthesis, *Eur. J. Clin. Invest. 10*, 27−35.
6. Salen, G., Shore, V., Tint, G. S., Forte, T., Shefer, S., Horak, I., Horak, E., Dayal, B., Nguyen, L., Batta, A. K., et al. (1989) Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis, *J. Lipid Res. 30*, 1319−1330.
7. Hubacek, J. A., Berge, K. E., Cohen, J. C., and Hobbs, H. H. (2001) Mutations in ATP-cassette binding proteins G5 (ABCG5) and G8 (ABCG8) causing sitosterolemia, *Hum. Mutat. 18*, 359−360.
8. Heimerl, S., Langmann, T., Moehle, C., Mauerer, R., Dean, M., Beil, F. U., von Bergmann, K., and Schmitz, G. (2002) Mutations in the human ATP-binding cassette transporters ABCG5 and ABCG8 in sitosterolemia, *Hum. Mutat. 20*, 151.
9. Klett, E. L., and Patel, S. (2003) Genetic defenses against noncholesterol sterols, *Curr. Opin. Lipidol. 14*, 341−345.
10. Brewer, H. B., Jr., and Santamarina-Fojo, S. (2003) New insights into the role of the adenosine triphosphate-binding cassette transporters in high-density lipoprotein metabolism and reverse cholesterol transport, *Am. J. Cardiol. 91*, 3E−11E.
11. Berge, K. E. (2003) Sitosterolemia: A gateway to new knowledge about cholesterol metabolism, *Ann. Med. 35*, 502−511.
12. von Bergmann, K., Sudhop, T., and Lutjohann, D. (2005) Cholesterol and plant sterol absorption: recent insights, *Am. J. Cardiol. 96*, 10D−14D.
13. Lammert, F., and Wang, D. Q. (2005) New insights into the genetic regulation of intestinal cholesterol absorption, *Gastroenterology 129*, 718−734.
14. Graf, G. A., Yu, L., Li, W. P., Gerard, R., Tuma, P. L., Cohen, J. C., and Hobbs, H. H. (2003) ABCG5 and ABCG8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion, *J. Biol. Chem. 278*, 48275−48282.
15. Klett, E. L., Lee, M. H., Adams, D. B., Chavin, K. D., and Patel, S. B. (2004) Localization of ABCG5 and ABCG8 proteins in human liver, gall bladder and intestine, *BMC Gastroenterol. 4*, 21.
16. Duan, L. P., Wang, H. H., and Wang, D. Q. (2004) Cholesterol absorption is mainly regulated by the jejunal and ileal ATP-binding cassette sterol efflux transporters Abcg5 and Abcg8 in mice, *J. Lipid Res. 45*, 1312−1323.
17. Small, D. M. (2003) Role of ABC transporters in secretion of cholesterol from liver into bile, *Proc. Natl. Acad. Sci. U.S.A. 100*, 4−6.
18. Yu, L., Hammer, R. E., Li-Hawkins, J., Von Bergmann, K., Lutjohann, D., Cohen, J. C., and Hobbs, H. H. (2002) Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion, *Proc. Natl. Acad. Sci. U.S.A. 99*, 16237−16242.
19. Yu, L., von Bergmann, K., Lutjohann, D., Hobbs, H. H., and Cohen, J. C. (2004) Selective sterol accumulation in ABCG5/ABCG8-deficient mice, *J. Lipid Res. 45*, 301−307.
20. Klett, E. L., Lu, K., Kosters, A., Vink, E., Lee, M. H., Altenburg, M., Shefer, S., Batta, A. K., Yu, H., Chen, J., Klein, R., Looije, N., Oude-Elferink, R., Groen, A. K., Maeda, N., Salen, G., and Patel, S. B. (2004) A mouse model of sitosterolemia: Absence of Abcg8/sterolin-2 results in failure to secrete biliary cholesterol, *BMC Med. 2*, 5.
21. Yu, L., Li-Hawkins, J., Hammer, R. E., Berge, K. E., Horton, J. D., Cohen, J. C., and Hobbs, H. H. (2002) Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol, *J. Clin. Invest. 110*, 671−680.
22. Wu, J. E., Basso, F., Shamburek, R. D., Amar, M. J., Vaisman, B., Szakacs, G., Joyce, C., Tansey, T., Freeman, L., Paigen, B. J., Thomas, F., Brewer, H. B., Jr., and Santamarina-Fojo, S. (2004) Hepatic ABCG5 and ABCG8 overexpression increases hepatobiliary sterol transport but does not alter aortic atherosclerosis in transgenic mice, *J. Biol. Chem. 279*, 22913−22925.
23. Yu, L., Gupta, S., Xu, F., Liverman, A. D., Moschetta, A., Mangelsdorf, D. J., Repa, J. J., Hobbs, H. H., and Cohen, J. C. (2005) Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion, *J. Biol. Chem. 280*, 8742−8747.
24. Higgins, C. F., and Linton, K. J. (2004) The ATP switch model for ABC transporters, *Nat. Struct. Mol. Biol. 11*, 918−926.
25. Holland, I. B., and Blight, M. A. (1999) ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans, *J. Mol. Biol. 293*, 381−399.
26. Lu, K., Lee, M. H., Yu, H., Zhou, Y., Sandell, S. A., Salen, G., and Patel, S. B. (2002) Molecular cloning, genomic organization, genetic variations, and characterization of murine sterolin genes Abcg5 and Abcg8, *J. Lipid Res. 43*, 565−578.
27. Graf, G. A., Cohen, J. C., and Hobbs, H. H. (2004) Missense mutations in ABCG5 and ABCG8 disrupt heterodimerization and trafficking, *J. Biol. Chem. 279*, 24881−24888.
28. Graf, G. A., Li, W. P., Gerard, R. D., Gelissen, I., White, A., Cohen, J. C., and Hobbs, H. H. (2002) Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface, *J. Clin. Invest. 110*, 659−669.
29. Urbatsch, I. L., Beaudet, L., Carrier, I., and Gros, P. (1998) Mutations in either nucleotide-binding site of P-glycoprotein (Mdr3) prevent vanadate trapping of nucleotide at both sites, *Biochemistry 37*, 4592−4602.
30. Lerner-Marmarosh, N., Gimi, K., Urbatsch, I. L., Gros, P., and Senior, A. E. (1999) Large scale purification of detergent-soluble P-glycoprotein from *Pichia pastoris* cells and characterization of nucleotide binding properties of wild-type, Walker A, and Walker B mutant proteins, *J. Biol. Chem. 274*, 34711−34718.
31. Urbatsch, I. L., Gimi, K., Wilke-Mounts, S., and Senior, A. E. (2000) Investigation of the role of glutamine-471 and glutamine-1114 in the two catalytic sites of P-glycoprotein, *Biochemistry 39*, 11921−11927.
32. Van Veldhoven, P. P., and Mannaerts, G. P. (1987) Inorganic and organic phosphate measurements in the nanomolar range, *Anal. Biochem. 161*, 45−48.
33. Goodno, C. C. (1982) Myosin active-site trapping with vanadate ion, *Methods Enzymol. 85* (Part B), 116−123.
34. Beaudet, L., Urbatsch, I. L., and Gros, P. (1998) High-level expression of mouse Mdr3 P-glycoprotein in yeast *Pichia pastoris* and characterization of ATPase activity, *Methods Enzymol. 292*, 397−413.
35. Cregg, J. M., Vedvick, T. S., and Raschke, W. C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*, *Biotechnology 11*, 905−910.
36. Urbatsch, I. L., Wilke-Mounts, S., Gimi, K., and Senior, A. E. (2001) Purification and characterization of N-glycosylation mutant

mouse and human P-glycoproteins expressed in *Pichia pastoris* cells, *Arch. Biochem. Biophys. 388*, 171−177.
37. Urbatsch, I. L., Tyndall, G. A., Tombline, G., and Senior, A. E. (2003) P-glycoprotein catalytic mechanism: Studies of the ADP-vanadate inhibited state, *J. Biol. Chem. 278*, 23171−23179.
38. Sankaran, B., Bhagat, S., and Senior, A. E. (1997) Inhibition of P-glycoprotein ATPase activity by beryllium fluoride, *Biochemistry 36*, 6847−6853.
39. Corvest, V., Sigalat, C., Venard, R., Falson, P., Mueller, D. M., and Haraux, F. (2005) The binding mechanism of the yeast F1-ATPase inhibitory peptide: Role of catalytic intermediates and enzyme turnover, *J. Biol. Chem. 280*, 9927−9936.
40. Harrison, M. A., Jones, P. C., Kim, Y. I., Finbow, M. E., and Findlay, J. B. (1994) Functional properties of a hybrid vacuolar H^+-ATPase in *Saccharomyces* cells expressing the Nephrops 16-kDa proteolipid, *Eur. J. Biochem. 221*, 111−120.
41. Rosa, M. F., and Sá-Correia, I. (1991) In vivo activation by ethanol of plasma membrane ATPase of *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol. 57*, 830−835.
42. Figler, R. A., Omote, H., Nakamoto, R. K., and Al-Shawi, M. K. (2000) Use of chemical chaperones in the yeast *Saccharomyces cerevisiae* to enhance heterologous membrane protein expression: High-yield expression and purification of human P-glycoprotein, *Arch. Biochem. Biophys. 376*, 34−46.
43. Sankaran, B., Bhagat, S., and Senior, A. E. (1997) Inhibition of P-glycoprotein ATPase activity by procedures involving trapping of nucleotide in catalytic sites, *Arch. Biochem. Biophys. 341*, 160−169.
44. Zhang, D. W., Graf, G. A., Gerard, R. D., Cohen, J. C., and Hobbs, H. H. (2006) Functional asymmetry of nucleotide-binding domains in ABCG5 and ABCG8, *J. Biol. Chem. 281*, 4507−4516.
45. Ramjeesingh, M., Li, C., Garami, E., Huan, L. J., Hewryk, M., Wang, Y., Galley, K., and Bear, C. E. (1997) A novel procedure for the efficient purification of the cystic fibrosis transmembrane conductance regulator (CFTR), *Biochem. J. 327* (Part 1), 17−21.
46. Mao, Q., Deeley, R. G., and Cole, S. P. (2000) Functional reconstitution of substrate transport by purified multidrug resistance protein MRP1 (ABCC1) in phospholipid vesicles, *J. Biol. Chem. 275*, 34166−34172.
47. Sun, H., Molday, R. S., and Nathans, J. (1999) Retinal stimulates ATP hydrolysis by purified and reconstituted ABCR, the photoreceptor-specific ATP-binding cassette transporter responsible for Stargardt disease, *J. Biol. Chem. 274*, 8269−8281.
48. http://humanabc.4t.com.
49. Smith, P. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. J., and Hunt, J. F. (2002) ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer, *Mol. Cell 10*, 139−149.
50. Chen, J., Lu, G., Lin, J., Davidson, A. L., and Quiocho, F. A. (2003) A tweezers-like motion of the ATP-binding cassette dimer in an ABC transport cycle, *Mol. Cell 12*, 651−661.
51. Urbatsch, I. L., Gimi, K., Wilke-Mounts, S., and Senior, A. E. (2000) Conserved walker A Ser residues in the catalytic sites of P-glycoprotein are critical for catalysis and involved primarily at the transition state step, *J. Biol. Chem. 275*, 25031−25038.
52. Tombline, G. B. L., Gimi, K., Tyndall, G. A., and Senior, A. E. (2004) Synergy between conserved ABC signature Ser residues in P-glycoprotein catalysis, *J. Biol. Chem. 279*, 5363−5373.
53. Urbatsch, I. L., al-Shawi, M. K., and Senior, A. E. (1994) Characterization of the ATPase activity of purified Chinese hamster P-glycoprotein, *Biochemistry 33*, 7069−7076.

BI0608055