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Bile Acids Stimulate ATP Hydrolysis in the Purified Cholesterol Transporter ABCG5/G8[†]

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ABSTRACT: ABCG5 and ABCG8 are half-size ABC transporters that function as heterodimers (ABCG5/G8) to reduce sterol absorption in the intestines and increase sterol excretion from the liver. Previous studies demonstrated that bile acids increased ABCG5/G8 specific cholesterol efflux in cell models. In this study we tested the effects of bile acids on ATP hydrolysis in *Pichia pastoris* purified ABCG5/G8 and found that they stimulated hydrolysis ~20-fold in wild-type ABCG5/G8 but not in a hydrolysis-deficient mutant. Nonconjugated cholate supported the highest ATPase activity in ABCG5/G8 (256 ± 9 nmol min^{-1} mg^{-1}). ATP hydrolysis was also stimulated by other conjugated bile acids and a mixture of bile acids resembling human bile with activities ranging from 129 ± 4 to 147 ± 14 nmol min^{-1} mg^{-1}. The kinetic parameters, inhibitor profiles, and lipid requirements of bile acid stimulated ATP hydrolysis were characterized. Cholate-stimulated ATP hydrolysis was maximal at concentrations of ≥10 mM MgATP and had a relatively high K_M (MgATP) of ~1 mM. Orthovanadate, BeFx, and AlFx effectively inhibited ABCG5/G8 at concentrations of 1 mM. Various lipid mixtures supported bile acid-stimulated ATP hydrolysis, which increased when cholesterol was present. The data demonstrate that bile acids together with lipids and cholesterol increase ATP hydrolysis in purified ABCG5/G8. Bile acids may promote an active conformation of purified ABCG5/G8 either by global stabilization of the transporter or by binding to a specific site on ABCG5/G8.

ABCG5 and ABCG8 are half-size ATP-binding cassette (ABC)[1] transporter proteins that function together as a heterodimer (ABCG5/G8) at the apical membranes of enterocytes and hepatocytes where this transporter pair limits the accumulation of cholesterol and other dietary sterols by transporting them into the intestinal lumen and bile (*1*−*3*). Mutations in either ABCG5 or ABCG8 cause sitosterolemia, a disease characterized by cholesterol and plant sterol accumulation in the blood and other tissues that results in premature atherosclerosis (*4*−*7*). Although sitosterolemia is rare, the discovery of ABCG5 and ABCG8 led to important new insights into whole body sterol regulation and provided evidence that specific molecular mechanisms regulate cholesterol absorption and excretion in the body (*8*). Disruption of either *abcg5* or *abcg8* in mice results in increased absorption and decreased excretion of cholesterol and other dietary sterols (*9*−*12*). Overexpression of the human genes in knockout mice decreased sterol absorption from the diet and increased plant sterol excretion into the bile (*13*). Recent studies confirmed that ABCG5/G8 directly transfers cholesterol and sitosterol in an ATP-dependent manner (*14*, *15*), but questions remain as to how the transporter pair utilizes ATP to facilitate the movement of cholesterol and other sterols across the membrane into the bile and intestinal lumen.

ABC transporters require the energy from ATP binding and hydrolysis in the nucleotide binding domains to drive substrate transport across the membrane, and ATP hydrolysis is typically stimulated by substrate binding to the transmembrane domains (*16*). We previously tested the effects of the ABCG5/G8 substrates cholesterol, sitosterol, and stigmasterol on ABCG5/G8 ATPase activity (*17*), but the specific activities were similar in the absence or presence of sterols. Other studies were also unable to demonstrate sterol stimulation of ABCG5/G8 ATPase activity, and one group showed that cholesterol and sitosterol slightly inhibited ATPase activity (*14*, *15*, *18*). Therefore, we hypothesized that ABCG5/G8 may require an acceptor for the hydrophobic sterol or a specific lipid/detergent environment for optimum ATP hydrolysis to occur.

ABCG5/G8 is expressed in locations where bile acids are present in high concentrations, and bile acids have been shown to increase ABCG5/G8 specific cholesterol efflux in cell models. Vrins et al. observed that cholesterol efflux by mouse Abcg5/g8 transfected in gallbladder epithelial cells required bile acids as an acceptor (*19*). Another study found that mixed micelles containing bile acids enhanced cholesterol and sitosterol efflux in a human colon carcinoma cell line (Caco-2) expressing ABCG5/G8 (*20*). Bile acids are water-soluble amphipathic molecules synthesized in the liver from cholesterol, transported across the canalicular membrane, and secreted into the bile. In addition to being synthesized from cholesterol, bile acids play a second

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[1]Abbreviations: ABC, ATP-binding cassette; NBDs, nucleotide binding domains; TMDs, transmembrane domains; PC, phosphatidylcholine; DDM, *n*-dodecyl β-D-maltopyranoside; OG, octyl β-D-glucoside; $AlCl_3$, aluminum chloride; NaF, sodium fluoride; $BeSO_4$, beryllium sulfate; Vi, orthovanadate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; SM, sphingomyelin; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine.

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important role in the elimination of cholesterol by solubilizing it in mixed phosphotidylcholine/bile acid micelles. Such mixed micelles serve as acceptors for the hydrophobic cholesterol and allow for its movement from the liver to the intestinal lumen for excretion (*21*, *22*).

Previously, we expressed human ABCG5/G8 in the yeast *Pichia pastoris* and purified the proteins for biochemical studies (*17*). Here, we investigated the effects of individual bile acids and a physiological mixture of bile acids on ATP hydrolysis in ABCG5/G8. We found that they stimulate ATP hydrolysis in wild-type ABCG5/G8 but not in a mutant protein. The bile acid stimulated ATP hydrolysis of ABCG5/G8 was characterized in terms of kinetic parameters, effects of inhibitors, and requirements for lipids and cholesterol.

EXPERIMENTAL PROCEDURES

Materials. *n*-Dodecyl β-D-maltopyranoside (DDM) was obtained from Inalco (Italy), and CHAPS and octyl β-D-glucoside (OG) were from Anatrace. ATP, bile acids (sodium salts), and aluminum chloride ($AlCl_3$) were from Sigma. Common chemicals including sodium fluoride (NaF), beryllium sulfate ($BeSO_4$), and orthovanadate (Vi) were from ThermoFisher. Liver lipids (Polar extract, catalog number 181108), *Escherichia coli* lipids (Polar extract, catalog number 100600), POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) (catalog number 850457), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) (catalog number 850757), POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine) (catalog number 840034), bovine PI (catalog number 840042), spingomyelin (catalog number 860062), and cholesterol (catalog number 700000) were from Avanti Polar Lipids (Alabaster, AL).

Expression of Human Wild-Type and Mutant ABCG5/G8. We previously coexpressed ABCG5 and ABCG8 both containing a 3C-protease peptide, a calmodulin binding peptide (CBP) tag, and a RGS-H_6 tag in *P. pastoris* (*17*) . To allow for true "co"-purification of the proteins, the ABCG5 gene was engineered to contain 12 histidines at the C terminus (ABCG5-RGSH_6GH_6), and both tags were removed from the ABCG8 construct and a STOP codon was inserted after the last amino acid (ABCG8-STOP). To facilitate cloning, a new *P. pastoris* expression vector pLIC-H6 was generated by removing the cleavage site for rhinovirus 3C protease and the calmodulin binding peptide (CBP) from pSGP18 (a derivative of pPICZB; Invitrogen) (*23*). pLIC-H6 contains a translational start site, sequences that allow for rapid insertion of reading frames via ligation-independent cloning (LIC), and a C-terminal six-His sequence (Figure S1, Supporting Information). Individual ORFs were amplified by PCR using PfuUltra II polymerase. PCR products were treated with T4 DNA polymerase in the presence of dGTP, incubated with cut and T4-treated vector (Figure S2, Supporting Information), and then transformed into XLI-blue cells (Novagen). The integrity of the individual ORFs in the expression plasmids was confirmed by DNA sequencing. We found a single nucleotide change at position 409 of the ABCG5 gene changing T137 to alanine; this error was corrected by site-directed mutagenesis restoring T137 as in the original published sequence (accession number NM_022436). Transformation and expression analysis were done as previously described (*17*). Briefly, 20 μg of pLIC-ABCG5-H_{12} and pLIC-ABCG8 (no tag) was linearized with *Pme*I and cotransformed into the *P. pastoris* strain KM71H by electroporation, and colonies were selected on 500 μg/mL zeocin. For protein expression analysis, several transformants were grown and induced with methanol in 10 mL cultures and microsomal membranes screened by Western blot for coexpression of ABCG5 and ABCG8 as described (*17*, *24*).

Construction of an ATP Hydrolysis Deficient Mutant of ABCG5/G8. The NBD of ABCG5 contains a conserved Walker A motif (GSSGSG**KT**T) while ABCG8 has two nonconserved residues in this motif (GSSGCG**RA**S, RA instead of KT or KS). Both the Walker A Lys and Ser/Thr are critical for ATP hydrolysis in other ABC transporters (*25*, *26*) and in ABCG5 (*14*, *15*, *18*, *27*). Therefore, replacing these residues in ABCG5 by their equivalent residues in ABCG8 should lead to a transporter with two degenerate NBDs that is incapable of ATP hydrolysis. The ABCG5 Walker A mutant K94A/T95A was generated with the QuikChange site-directed mutagenesis kit (Stratagene) using pLIC-ABCG5-H_{12} as a template and the forward oligonucleotide 5′-CTCAGGCTCCGGAAGAGCCACGCTGCTGGAC and the reverse oligonucleotide 5′-GTCCAGCAGCGTGGCTCTT-CCGGAGCCTGAG. The combined mutant (ABCG5KT-RA/G8) was created by cotransforming 20 μg of mutant ABCG5 and wild-type ABCG8 DNA into *P. pastoris* following the same procedures as for wild type.

Purification of ABCG5/G8. Wild-type and mutant ABCG5/G8 were purified from fermentor cultures of *P. pastoris* as described for previous ABCG5/G8 and Pgp purification procedures (*17*, *24*) with the following modifications: Two 100 g batches of *P. pastoris* cells expressing wild-type or mutant ABCG5/G8 were disrupted in a glass bead beater as described (*17*), except 10 mM DTT was included during cell breakage to fully reduce the proteins. All buffers for membrane preparation and chromatography were supplemented with 1 mM β-mercaptoethanol and 0.1 mM tris(2-carboxyethyl)phosphine (TCEP) to keep the proteins reduced. Microsomal membranes were solubilized for 30 min at 4 °C in 0.5% *n*-dodecyl β-D-maltoside (DDM) at a final protein concentration of 2 mg/mL in "buffer A" [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM imidazole, 20% glycerol, 1 mM β-mercaptoethanol, and 0.1 mM TCEP] and protease inhibitors as described (*17*). The soluble supernatant was chromatographed on 15 mL Ni-NTA superflow resin (Qiagen) in buffers containing 0.1% DDM as described (*17*), except the resin was washed with 20 bed volumes of buffer A (with glycerol reduced to 10%) followed by 3 bed volumes of buffer A containing 20 mM imidazole. Proteins were eluted from the resin using 3 bed volumes of 200 mM imidazole buffer [50 mM Tris-HCl (pH 7.4), 10% glycerol, 200 mM imidazole, and 0.1% DDM] and concentrated to ≤15 mL by ultrafiltration using an Amicon Ultra centrifugal filter with a YM-100 membrane (Millipore). For the next step, 15 mL of Whatman DE52 cellulose was charged with 5 bed volumes of 0.5 M Tris−acetate (pH 6) and then equilibrated with 20 bed volumes of 10 mM Tris−acetate (pH 6) followed by 2 bed volumes of buffer BB [10 mM Tris−acetate (pH 6), 10% glycerol, 0.1% DDM]. The concentrated 200 mM imidazole eluate from the Ni-NTA column was diluted with 4 volumes of buffer BB and applied to the DE-52 column at 4 °C. The flow-through was collected, and the resin was washed again with 2 bed volumes of buffer BB. The effluent was combined with the flow-through fraction and concentrated by ultrafiltration to 3−5 mL. To retrieve more tightly bound ABCG5-H_{12}/G8, the DE-52 column was eluted with 2 bed volumes of buffer BB containing 50 mM NaCl and concentrated as above. Protein concentrations were determined by

UV spectroscopy using a calculated A_{280} of 1.05 mg/mL for ABCG5/G8. The concentrated material was aliquoted and stored at −80 °C. The yield was 2−4 mg of purified protein per 200 g of cells for wt ABCG5/G8 and 1.3−2.3 mg for the mutant.

Lipid and Bile Acid Preparation. Lipid stocks were prepared in 50 mM Tris-HCl buffer, pH 7.4, from liver lipids or *E. coli* lipids to a final concentration of 50 mg/mL (65 mM) by sonicating in a Bransonic 151OR-MT water bath sonicator for 30 min at room temperature as described (*24*). Bile acids (sodium salts) were dissolved at 195 mM in water (pH 7.4) and were mixed with an equal volume of lipid by brief vortexing to give a 3:1 molar ratio of bile acid to lipid. These bile acid concentrations readily dissolved the lipids, resulting in a clear and homogeneous solution at room temperature. Lipid stocks and bile acid/lipid mixtures were stored frozen at −20 °C under inert nitrogen gas. For the physiological bile acid mixture, glycochenodeoxycholate, taurochenodeoxycholate, glycocholate, taurochoalte, glycodeoxycholate, and taurodeoxychoalte were mixed at a molar ratio of 21:19:24:16:13:7 (*21*, *28*). For the lipid mixture, POPC, POPE, POPS, PI, sphingomyelin (SM), and cholesterol were prepared and mixed at a weight ratio of 46:22:6:6:16:4 as described (*29*) .

ATPase Assays. Protein in 0.1% DDM was mixed with 20 mM DTT for 2 min on ice and then activated with the indicated 2× bile acid/lipid mixture for 5 min at room temperature. Reactions were carried out in a final volume of 50 μL containing 50 mM Tris-HCl (pH 7.4), 20 mM $MgSO_4$, 20 mM NaATP, and 10 mM sodium azide at 37 °C for appropriate times. Reactions were started by addition of 5−10 μg of activated ABCG5/G8 (typically in a volume of 25 μL) to 25 μL of 2× ATP cocktail and stopped by the addition of 1 mL of 20 mM ice-cold H_2SO_4 containing 0.05% DDM to reduce background during the malachite green development. Released inorganic phosphate was assayed by the method of Van Veldhoven and Mannaerts (*30*). Azide was included in the assay to inhibit F_1-ATPase activity. However, virtually identical results were obtained in the absence or presence of sodium azide, indicating that the purified ABCG5/G8 is essentially free of any contamination by F_1-ATPase activity. For the determination of kinetic parameters, MgATP concentrations were varied with an excess of 2 mM free Mg^{2+} over MgATP. The presence of free Mg^{2+} is important because bile acids bind magnesium. Cholate-stimulated ATPase activity in the presence of a 10 mM excess of Mg^{2+} over MgATP (total 30 mM $MgSO_4$) was reduced by 34%. This indicated that a large excess of Mg^{2+} is inhibitory and that Mg^{2+} does not become limiting in the assay through binding to cholate. When high bile acid (sodium salt) concentrations were used, the ionic strength of the buffer was also high. To verify that these salt concentrations did not interfere with hydrolysis, we assayed ATPase in the presence of 50 or 100 mM sodium chloride and found that these salt concentrations had no effect on activity (< 3% difference). Inhibition was assayed in cocktails containing 20 mM MgATP supplemented with either 0.5−1 mM Vi, 0.5−1 mM $BeSO_4$, and 5 mM NaF (BeFx) or 0.5−1 mM $AlCl_3$ and 5 mM NaF (AlFx). Orthovanadate solutions (100 mM) were prepared from Na_3VO_4 at pH 10 and boiled for 2 min before each use to break down polymeric species (*31*).

Routine Procedures. SDS−PAGE was performed using criterion gels (Bio-Rad). Samples were dissolved in ≥0.3 volumes 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 10 min at room temperature and then resolved on 10% polyacrylamide gels. Western blot analysis followed standard procedures and used a monoclonal anti-RGS-His_4 antibody (Qiagen), a polyclonal anti-ABCG8 antibody (Novus), and a polyclonal anti-F_1-ATPase antibody (Agrisera, Sweden). The software SigmaPlot 11.0 was used for nonlinear regression analysis.

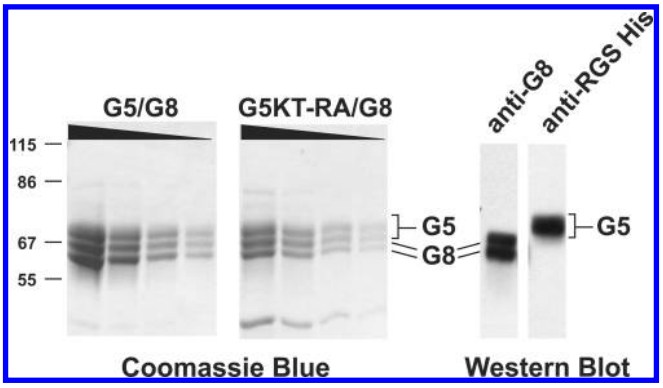

FIGURE 1: Wild-type and mutant human ABCG5/G8 purified from *P. pastoris*. ABCG5 containing a C-terminal RGS-H_{12} tag and ABCG8 (no tag) were coexpressed in the yeast *P. pastoris*, and the proteins were copurified as described under Experimental Procedures. Decreasing concentrations (10, 5, 2.5, and 1.25 μg) of the final purified, wild-type ABCG5/G8 (G5/G8) and the mutant, ABCG5-K94A/T95A coexpressed with wild-type ABCG8 (G5KT-RA/G8), were resolved by SDS−PAGE followed by Coomassie Blue staining (left). ABCG5 and ABCG8 resolve into three bands because of differential glycosylation (*17*). The positions of the MW protein markers are indicated in kDa. Western blot analysis (right) using a polyclonal anti-ABCG8 and a monoclonal anti-RGS-His_4 antibody (which recognizes the His tag on ABCG5) confirmed the presence of ABCG5 and ABCG8.

RESULTS

Purification of Human ABCG5/G8. Previously, we coexpressed human *ABCG5* and *ABCG8* in the yeast *P. pastoris* for large-scale purification of the proteins. However, very small amounts of the mitochondrial F_1-ATPase copurified in our preparations after tandem-affinity chromatography on Ni-NTA and calmodulin resins (*17*). To improve the purification procedure, we created new ABCG5 and ABCG8 constructs to express in *P. pastoris*. Twelve histidines were added to the C terminus of ABCG5 to increase its binding affinity for the Ni-NTA resin. This new ABCG5 construct was coexpressed with nontagged ABCG8 in *P. pastoris* to allow for "true" copurification of the proteins. Coexpressed ABCG5 and ABCG8 (termed ABCG5/G8) were purified by affinity chromatography on Ni-NTA resin followed by anion-exchange chromatography on DE52 resin as described in Experimental Procedures. The His_{12} tag allowed for more stringent washes of the Ni-NTA resin with low concentrations of imidazole (10 and 20 mM) to remove contaminating proteins. The final material is > 85% pure, and F_1-ATPase was not detectable by Western blot even when large amounts of protein (10 μg/lane) were analyzed (Figure S3 of Supporting Information). Nontagged ABCG8 efficiently copurified with ABCG5 confirming a tight association of the two proteins during solubilization and chromatography (Figure 1). ABCG5 homodimers/oligomers may have copurified using this strategy (the His tag is on ABCG5); however, ABCG5 homodimers were less stable in previous purifications, and their yield was ~5-fold lower than the yield for coexpressed ABCG5/G8 heterodimer (*17*).

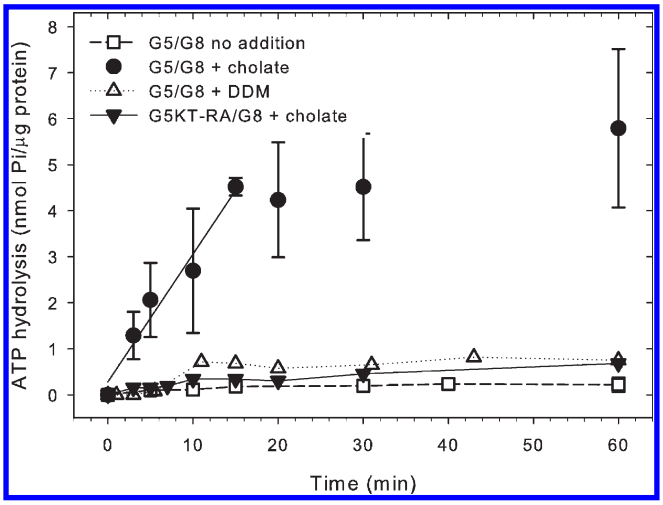

FIGURE 2: Cholate stimulates ATP hydrolysis in wild-type but not mutant ABCG5/G8. Purified, detergent-soluble wild-type ABCG5/G8 was activated with liver lipids alone or with liver lipids containing 1.5% (34.8 mM) cholate or 0.25% (4.9 mM) DDM. Purified, mutant ABCG5/G8 (G5KT-RA/G8) was activated with liver lipids containing 1.5% (34.8 mM) cholate. Both mutant and wild-type ABCG5/G8 were assayed in cocktails containing 20 mM MgATP and 10 mM sodium azide for the indicated times at 37 °C. The details are given under Experimental Procedures. Wild-type ABCG5/G8 no addition (n = 10) and +cholate (n = 4) data points represent the mean ± standard deviations, and all other data points represent the means of duplicate experiments ± range; where not visible, error bars are smaller than the plot symbol.

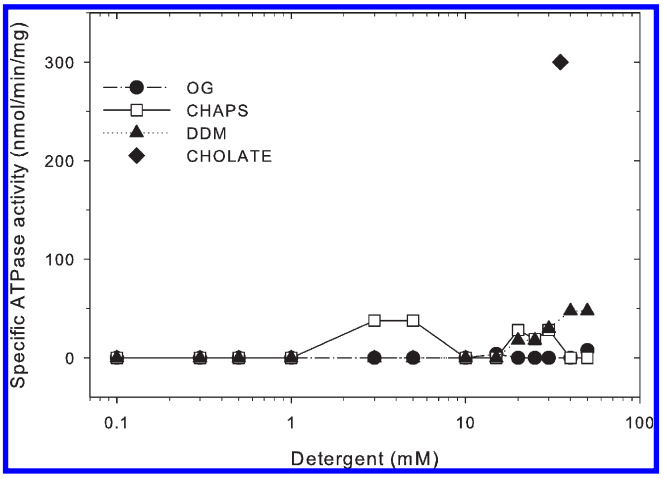

FIGURE 3: Effects of common detergents on the ABCG5/G8 ATPase activity. Purified ABCG5/G8 was assayed as in Figure 2 except increasing concentrations of *n*-octyl β-D-glucoside (OG), CHAPS, and DDM were included. Each data point represents the mean from at least two independent experiments ± range; error bars were very small and are contained within the symbols. ABCG5/G8 ATPase activity in the presence of 1.5% (34.8 mM) cholate was used as a control in these experiments (300 ± 122 nmol min^{-1} mg^{-1}, n = 30) and is represented as the mean ± standard deviation.

Cholate Stimulates ATP Hydrolysis in Wild-Type But Not Mutant ABCG5/G8. The specific ATPase activity of ABCG5/G8, purified following our revised procedure, was between 10 and 20 nmol min^{-1} mg^{-1}. ATPase activity was the same in the presence or absence of sodium azide, a known inhibitor of F_1-ATPase, indicating F_1-ATPase is not present in our purified preparations. This activity is similar to what we previously reported for double-tagged ABCG5/G8 (15 ± 5 nmol min^{-1} mg^{-1}) (*17*), and it is relatively low compared to native mouse ABCG5/G8 purified from mouse livers (390 ± 50 nmol min^{-1} mg^{-1}) as reported by the Hobbs/Xie group (*15*). Furthermore, the ATP hydrolysis rate of wild-type ABCG5/G8 was similar to values observed in the ABCG5KT-RA/G8 mutant (7.6 ± 0.3), suggesting that under these assay conditions very little hydrolysis occurred in the *P. pastoris* purified ABCG5/G8.

Bile acids are associated with cholesterol elimination from the body (see Discussion), and two studies demonstrated that bile acids increased ABCG5/G8 specific cholesterol efflux in cell models (*19*, *20*). Therefore, we tested the effects of the bile acid cholate on the ATPase activity of purified ABCG5/G8. The detergent-soluble protein was preincubated with 0.5% liver lipids in the presence of 1.5% cholate, and ATP hydrolysis was assayed over a period of 1 h at 37 °C (Figure 2). We found that, in the presence of cholate, ATP hydrolysis in ABCG5/G8 increased linearly during the first 15 min of the assay and then subsequently slowed, possibly due to accumulation of ADP product. Initial rates of ABCG5/G8 ATP hydrolysis were 300 ± 122 nmol min^{-1} mg^{-1} (n = 30), which is a 15−30-fold increase in ATP hydrolysis compared to rates measured in the absence of cholate. Additionally, while these results show that cholate significantly stimulated wild-type ABCG5/G8 ATP hydrolysis, the ABCG5KT-RA/G8 mutant showed very low ATP hydrolysis in the presence or absence of this bile acid (Figure 2).

Cholate is also used as a detergent for membrane protein solubilization, and thus, we investigated whether the stimulation of ATP hydrolysis was caused by a detergent effect due to changes in the fluidity of the protein/lipid complex in the assay. The ATP hydrolysis was not affected by preincubation of ABCG5/G8 with liver lipids and 0.25% *n*-dodecyl β-D-maltopyranoside (4.9 mM DDM, ~27 times its CMC), a concentration that completely dissolved the liver lipids, similar to 1.5% cholate (~4 times its CMC) (Figure 2). We further studied the concentration dependence of DDM and another nonionic detergent, *n*-octyl β-D-glucoside (OG), as well as the zwitterionic detergent CHAPS, which has a core structure similar to cholate (Figure 3). ATP hydrolysis was not significantly stimulated by any of these detergents over a range of concentrations (from 0.1 to 50 mM). Since ABCG5/G8 was purified in DDM, we tested whether this detergent affects cholate-stimulated ATP hydrolysis. Increasing concentrations of DDM inhibited ATP hydrolysis with half-maximal inhibition at ~10 mM DDM in the presence of 20 mM cholate (Figure S4 of Supporting Information). The data demonstrate that DDM does not support ATP hydrolysis in ABCG5/G8 and suggest that cholate may stimulate hydrolysis by competing with DDM (see Discussion).

Dependence of ABCG5/G8 ATPase Activity on the Concentration of Bile Acids. The effects of bile acids on ABCG5/G8 were further investigated by measuring ATP hydrolysis in the presence of the three major physiologically relevant conjugated forms of the bile acids found in human bile: cholate, chenodeoxycholate, and deoxycholate (*21*). For these experiments, bile acids were mixed with liver lipids at a molar ratio of 3:1 to resemble the average ratio of bile acids to phospholipids in human bile (*32*). ABCG5/G8 was preincubated with the bile acid/lipid mixture immediately before initiating the hydrolysis reaction, and ATP hydrolysis was measured over a wide range of bile acid concentrations (0.1 mM up to 50 mM, maintaining a bile acid to lipid ratio of 3:1) to determine the level of stimulation and the affinity of ABCG5/G8 for individual bile acids (see Experimental Procedures). In Figure 4A, unconjugated cholate stimulated the ATPase activity in a concentration-dependent manner

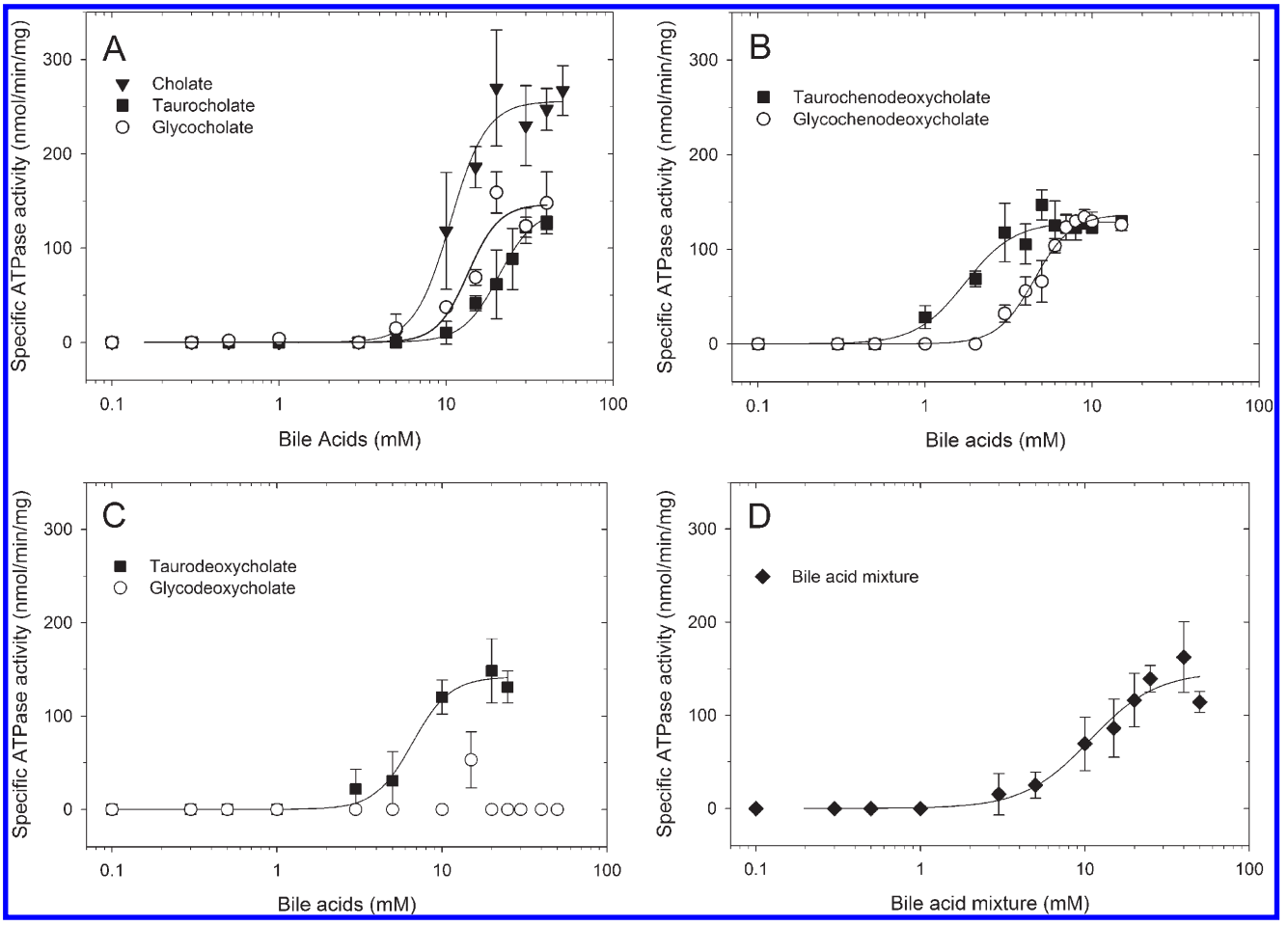

FIGURE 4: Concentration dependence of bile acids on the ABCG5/G8 ATPase activity. (A) Purified ABCG5/G8 was assayed for 15 min at 37 °C as in Figure 2 in the presence of increasing concentrations of cholate or its conjugates (taurocholate or glycocholate) as indicated. The concentration dependence of the conjugates of chenodeoxycholate and deoxycholate are shown in (B) and (C). A physiological mixture of conjugated bile acids that resembles human bile (40:40:20 chenodeoxychoalte:cholate:deoxycholate; see Experimental Procedures) was assayed as in (D). The solid lines are fits to the Hill equation (r^2 values range from 0.94 to 0.99); the steep slopes of the curves with Hill coefficients between 2 and 4.6 (see Table 1) indicate strong cooperativity. Each data point in (A) and (C) represents the mean from at least two independent experiments ± range; each data point in (B) and (D) represents the mean ($n = 3$) ± standard deviation.

Table 1: Dependence of ABCG5/G8 ATPase Activity on the Concentration of Bile Acids

bile acid	maximal ATPase activity (nmol min^{-1} mg^{-1})[a]	half-maximal stimulation (mM)[b]	maximum stimulation (mM)[b]	Hill coeff[c]
none	9.2 ± 7	N/A	N/A	N/A
cholate	256 ± 9	11	20	4.1
taurocholate	142 ± 11	21	40	4.0
glycocholate	147 ± 14	14	30	4.6
taurochenodeoxycholate	129 ± 4	2	5	2.9
glycochenodeoxycholate	137 ± 6	5	8	3.9
taurodeoxycholate	142 ± 6	7	20	3.8
glycodeoxycholate	53 ± 42	N/A	N/A	N/A
mixture	147 ± 13	11	30	2

[a]The standard errors (SE) were calculated from the fits shown in Figure 4 using SigmaPlot 11. [b]Concentration required for half-maximal stimulation and maximal stimulation of ATPase activity. [c]Hill coefficients were obtained from fits to the Hill equation in Figure 4.

with half-maximal activity at 11 mM and maximal activity at ≥20 mM. The conjugated bile acids tauro- and glycocholate (Figure 4A), tauro- and glyco**chenodeoxy**cholate (Figure 4B), as well as tauro**deoxy**cholate (Figure 4C) all effectively stimulated ATPase activity, except for glycodeoxycholate (Figure 4C). In fact, glycodeoxycholate was the only bile acid that did not stimulate hydrolysis, and the reason for this selectivity is unclear. The highest specific ATPase activity in these experiments was observed with cholate, followed by glycocholate, taurocholate, taurodeoxycholate, glycochenodeoxycholate, and taurochenodeoxycholate (see Table 1). Overall, maximal ATPase activities were observed at relatively high concentrations of at least 5 mM (taurochenodeoxycholate) and 10−20 mM for the others. However, these high concentrations are within the range of bile acid concentrations found in human bile (2−45 mM) (*32*). Furthermore, the concentrations required for half-maximal stimulation

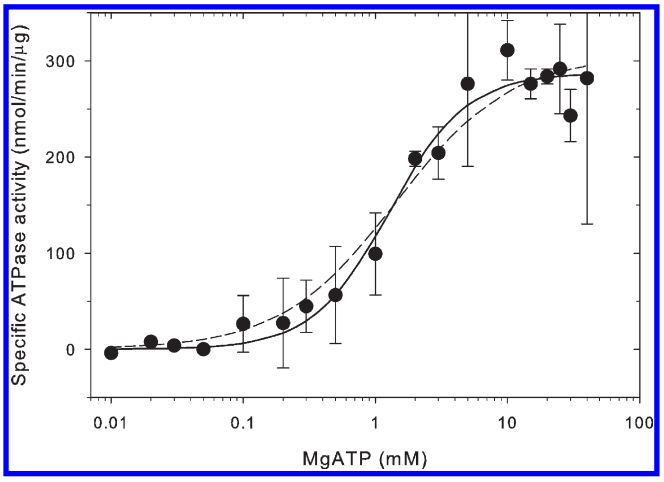

FIGURE 5: MgATP concentration dependence of cholate-stimulated ABCG5/G8 ATP hydrolysis. Purified ABCG5/G8 was activated with liver lipids and cholate (20 mM, 0.9%) to maximally stimulate activity and then assayed in cocktails containing increasing concentrations of MgATP for 10 min at 37 °C; see Experimental Procedures. The solid line is a fit to the Hill equation, which gives a better fit with an r^2 value of 0.98 than the Michaelis−Menten equation with an r^2 value of 0.97 (dashed line). The figure shows the mean ± range from two independent experiments.

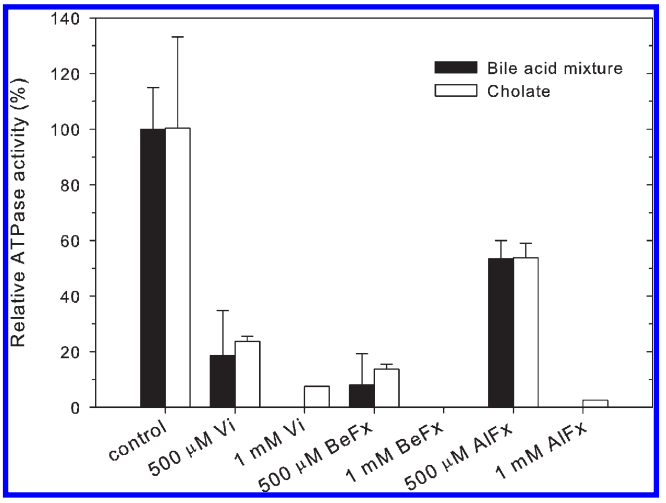

FIGURE 6: Inhibition of ABCG5/G8 ATP hydrolysis. Purified ABCG5/G8 was activated with liver lipids containing either 20 mM bile acid mixture (see Figure 4D) or 20 mM cholate, and ATP hydrolysis was assayed for 10 min at 37 °C. The reaction cocktails contained phosphate analogue inhibitors at a final concentration of 500 μM or 1 mM. One hundred percent activity (in the absence of inhibitor) was 126 ± 42 nmol min^{-1} mg^{-1} for the bile acid mixture and 268 ± 41 nmol min^{-1} mg^{-1} for cholate. The figure shows the means ± range from two independent experiments.

were close to the concentrations for maximum activity (2−3-fold difference, Table 1), which is evident from the steep slopes of the curves with Hill coefficients between 3 and 4.6. This may indicate cooperativity between bile acids and the ATPase function of ABCG5/G8. The steep increase in activity appears to correlate with the bile acids' CMCs, which suggests either stimulation by the micellar form or the absence of free bile salt molecules at the low concentration range due to absorption into the lipid phase. In summation, these data show that bile acids greatly enhance ATP hydrolysis in purified ABCG5/G8.

Next, we tested the effects of a bile acid mixture resembling the physiological composition of human bile on ABCG5/G8 ATP hydrolysis (Figure 4D). Cholate, chenodeoxycholate, and deoxycholate are present at a molar ratio of approximately 40:40:20 in human bile with the majority (~60%) conjugated to glycine and the remainder (~40%) conjugated to taurine (*21*, *28*). In Figure 4D, the "bile acid mixture" was prepared at these ratios with conjugated bile acids and then mixed with liver lipids at a molar ratio of 3:1. ABCG5/G8 was preincubated with this mixture immediately before initiating the hydrolysis reaction, and ATP hydrolysis was measured over a wide range of bile acid concentrations (0.1 mM up to 50 mM; see Experimental Procedures). Maximal ATPase activity was 147 ± 13 nmol min^{-1} mg^{-1} and occurred at ~20 mM bile acid mixture (see Table 1). The data indicate that the physiological bile acid mixture was as effective as or better than individual bile acid conjugates, but the ATPase activity was not quite as high as observed in the presence of nonconjugated cholate.

Kinetics of ABCG5/G8 ATPase Activity. Kinetic parameters of cholate-stimulated ATP hydrolysis were determined from multiple experiments after activation of the purified proteins with liver lipids and 20 mM cholate. We observed maximal ATP hydrolysis by ABCG5/G8 at concentrations of ≥10 mM MgATP with a V_{max} of 287 ± 9 nmol min^{-1} mg^{-1} and a K_m(MgATP) of ~1 mM (Figure 5). The best fit of the data was obtained using the Hill equation (Figure 5, solid lines) with a Hill coefficient of 1.5, indicating cooperativity between the two NBDs for ATP hydrolysis as previously reported.

Inhibition of the ABCG5/G8 ATPase by Transition- and Ground-State Analogues. Transition- and ground-state analogues have been used extensively to examine the catalytic mechanism of P-glycoprotein and other ABC transporters (*33*, *34*). Therefore, we tested these analogues for their ability to inhibit bile acid stimulated ABCG5/G8 ATP hydrolysis. Purified ABCG5/G8 was activated with liver lipids containing either 20 mM bile acid mixture (as in Figure 4D) or 20 mM cholate, and ATP hydrolysis was assayed in cocktails containing phosphate analogue inhibitors at a final concentration of 500 μM or 1 mM. Orthovanadate (Vi) and BeFx abolished ATP hydrolysis at concentrations of 1 mM (Figure 6). AlFx was not quite as effective as BeFx at a concentration of 500 μM but still completely inhibited ABCG5/G8 at 1 mM. Previously, we showed ATP hydrolysis in ABCG5/G8 was not Vi sensitive, but cholate-stimulated ATP hydrolysis in ABCG5/G8 was inhibited by Vi (*17*).

Lipid and Cholesterol Dependence on ABCG5/G8 ATPase Activity. We tested different lipid extracts and synthetic lipids for their ability to support ATP hydrolysis in purified ABCG5/G8. In this analysis, extracts from liver lipids (containing ~4% cholesterol), a synthetic lipid mixture resembling the composition of liver plasma membrane lipids (POPC:POPE:POPI:POPS:SM:Chol at a weight ratio of 46:22:6:6:16:4) (±4% cholesterol) (*14*), *E. coli* lipids (±cholesterol), and POPC lipids (±cholesterol) were included. Purified ABCG5/G8 was preincubated with 20 mM cholate or 20 mM bile acid mixture (see Figure 4D) and the indicated lipids (at a molar ratio of 3:1) and assayed for ATP hydrolysis. All lipids, including POPC alone, supported ATP hydrolysis in ABCG5/G8, indicating that ABCG5/G8 may not have particular lipid requirements. Preincubation of ABCG5/G8 with liver lipids resulted in the highest activities, while preincubation with synthetic liver plasma membrane and *E. coli* lipids, devoid of cholesterol, resulted in the lowest (Figure 7). Interestingly, the addition of cholesterol in all lipids enhanced ATP hydrolysis in ABCG5/G8.

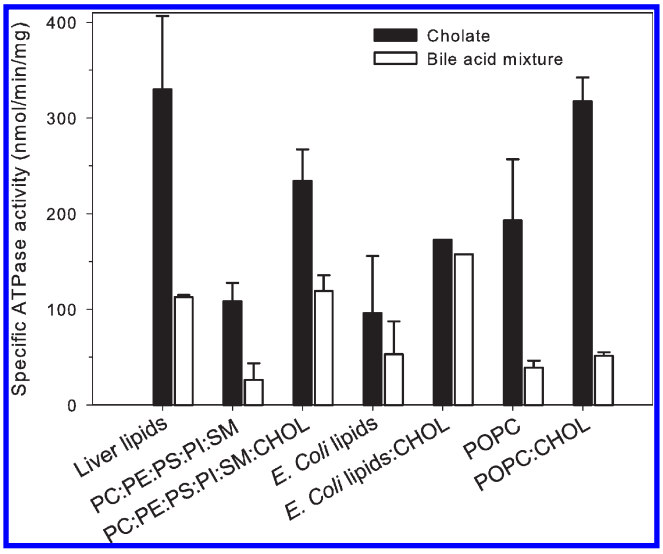

FIGURE 7: Lipid dependence of cholate-stimulated ABCG5/G8 ATP hydrolysis. Purified ABCG5/G8 was activated with either liver lipids, a lipid mixture (POPC:POPE:POPI:POPS:SM:CHOL at a ratio of 46:22:6:6:16:4) in the absence or presence of 4% cholesterol (CHOL), *E. coli* lipids (±10% CHOL), or POPC lipids (±8% CHOL) as indicated containing either 20 mM cholate or 20 mM bile acid mixture (see Figure 4D). The details are given under Experimental Procedures. ATP hydrolysis was assayed for 15 min at 37 °C as in Figure 2. The figure shows the means ± range from two independent experiments.

DISCUSSION

In this study, we show for the first time that bile acids stimulate ATP hydrolysis in the human ABCG5/G8 sterol transporter. We demonstrate that ABCG5/G8, purified from the yeast *P. pastoris*, has very low ATPase activity in detergent or liver lipid solutions, but in the presence of lipids and the bile acid cholate, the ATPase activity increased ~20-fold (Figure 2) to values previously observed for native ABCG5/G8 (no tag) purified from mouse livers (*15*). In contrast, the purified ABCG5KT-RA/G8 mutant displayed the same very low ATPase activity when assayed in the presence or absence of cholate. We further demonstrate that the major bile acids in humans (cholate, chenodeoxycholate, and deoxycholate) conjugated with glycine or taurine stimulated ATP hydrolysis in wild-type ABCG5/G8 when assayed individually (Figure 4A−C) or as a mixture resembling the composition of human bile (Figure 4D). Nonconjugated cholate supported the highest ATPase activities in ABCG5/G8 (256 ± 9 nmol min^{-1} mg^{-1}) compared to other conjugated bile acids and the bile acid mixture (129 ± 4 to 147 ± 14 nmol min^{-1} mg^{-1}, Table 1). The concentrations required for maximum activity were relatively high (in the millimolar range) but were within the range of concentrations found in the intestinal lumen and human bile (10−50 mM) where ABCG5/G8 are expressed (*32*).

It is not clear how bile acids stimulate ATP hydrolysis in ABCG5/G8. The simplest explanation for the enhanced ATPase activity is that liver lipids and bile acids promote an active conformation of ABCG5/G8, possibly through interactions with the hydrophobic TMDs and/or the extracellular loops. Bile acids may stabilize ABCG5/G8 through several different ways. The proteins were purified in DDM, a detergent commonly used for purification of membrane proteins in active conformations (*35*), but DDM may interact unfavorably with ABCG5/G8 and keep the protein in an inactive form. In order to investigate this possibility, we added DDM to cholate-stimulated ABCG5/G8 and found that DDM (≥10 mM) did inhibit cholate-stimulated ABCG5/G8 ATP hydrolysis (Supporting Information Figure S4). This may indicate that cholate stimulates purified ABCG5/G8 by competing with bound DDM to reverse inhibition, or the addition of DDM may change the bile acid mixed micelles so that they no longer effectively stimulate ATP hydrolysis. Alternatively, bile acids could affect the fluidity of the protein/lipid complex thereby promoting activity. To test this possibility, we assayed ABCG5/G8 in the presence of the detergents OG and CHAPS, which has a sterol core similar to bile acids. These detergents did not stimulate hydrolysis in ABCG5/G8 (Figure 3), suggesting that the bile acid stimulation was not a simple detergent effect but possibly a specific interaction of bile acids with ABCG5/G8. Purification of native mouse ABCG5/G8 involved elution of the proteins from a Blue2 affinity column with high concentrations (2%) of cholate and subsequent exchange of the protein into a nonionic detergent ($C_{12}E_9$). This protein preparation displayed the highest activity reported to date (*15*), which may suggest that tightly bound cholate copurified with ABCG5/G8 and acts as a chemical chaperone to stabilize this transporter pair. Thus, cholate and other bile acids may be considered as detergents or additives for future purifications of ABCG5/G8 and perhaps other ABC transporters that reside in the canalicular duct.

Another interesting possibility is that bile acid interactions with ABCG5/G8 may be important for its function *in vivo*. ABCG5/G8 is expressed in locations where bile acids are present in high concentrations above their critical micelle concentration (CMC, ~10 mM), and mixed micelles are readily formed. Mixed micelles made up of bile acids and phospholipids have been reported to act as acceptors for cholesterol, which is a very hydrophobic molecule, to allow for its transport in the bile and the intestine for eventual elimination (*21*). Two studies have demonstrated that bile acids increased ABCG5/G8 specific cholesterol efflux in cell models by serving as cholesterol acceptors, in contrast to other known cholesterol acceptors (i.e., apolipoprotein A-I and high-density lipoprotein HDL) that were not able to increase efflux (*19*, *20*). The authors suggested that cholesterol transported by ABCG5/G8 remains bound to the protein until it is picked up by mixed micelles or that bile acid monomers may compete with bound cholesterol on the transporter with the micelles merely serving as a "sink" to carry the cholesterol away. We hypothesize that mixed micelles might enhance ABCG5/G8 activity by docking onto the extracellular loops or TMDs to induce conformational changes that stimulate ATP hydrolysis and mimic ABCG5/G8's native surroundings in the bile-canalicular duct. However, we cannot exclude the possibility that bile acid monomers may bind to the cholesterol-binding site within the TMDs of ABCG5/G8 along with cholesterol to aid in its movement off the transporter and thereby stimulate ATP hydrolysis. The recent crystal structure of P-glycoprotein, which transports hydrophobic drugs as well as cholesterol (*34*), revealed that this related ABC transporter is capable of binding two (or more) substrate molecules simultaneously within its large polyspecific substrate binding site (*35*). Future sterol transfer studies with mixed bile acid/phospholipid micelles or bile acid monomers will be needed to evaluate possible micelle interactions or cotransfer of these molecules by ABCG5/G8.

We further characterized the kinetic parameters, inhibitor profiles, and lipid requirements of bile acid stimulated ATP hydrolysis. ATPase activity of ABCG5/G8 was highest in the

presence of cholate, and thus, we selected cholate and the physiological bile acid mixture for these experiments. The cholate-stimulated ATP hydrolysis was maximal at concentrations of ≥10 mM MgATP and had a relatively high K_M(MgATP) of ~1 mM (Figure 5), which is common in ABC transporters (*33*) and may indicate that the proteins are sensitive to the energy state of the cell (*19*). Orthovanadate (Vi), BeFx, and AlFx effectively inhibited ABCG5/G8 at concentrations of 1 mM (Figure 6). Vi has been reported to inhibit ATP hydrolysis of ABCG5/G8 in insect cell membranes as well as cholesterol transfer by purified, reconstituted ABCG5/G8 (~90%) (*18*, *29*), but it was less effective at inhibiting ATP hydrolysis in purified ABCG5/G8 (*17*, *29*). The reasons for the latter are not clear, but hydrolysis may not have been assayed under optimal conditions.

Lipids are a crucial factor in maintaining the functional conformations of membrane proteins, and therefore we tested bile acid stimulation of ATP hydrolysis in combination with various lipids to investigate the lipid requirements of ABCG5/G8. All lipids, even POPC alone, supported ATP hydrolysis with the highest activity observed in the presence of liver lipid extracts (Figure 7). The most intriguing observation was the effect of cholesterol on the bile acid stimulated ATP hydrolysis. When cholesterol was present in the lipid mixtures, an obvious increase in bile acid stimulated ATP hydrolysis occurred. While it is not clear whether cholesterol-enhanced stimulation of activity is due to an increase in stabilization of the purified proteins or due to cholesterol directly interacting with ABCG5/G8, it is the first time that cholesterol has been shown to positively affect ATP hydrolysis of ABCG5/G8.

In conclusion, these studies provide evidence that bile acids stimulate ATP hydrolysis in purified ABCG5/G8. Bile acids may promote an active conformation and may be useful detergents for future purifications and functional studies of ABCG5/G8. Furthermore, the bile acid stimulation of this sterol transporter may provide a basis for further studies on bile acid regulation of cholesterol transport and on their possible use in cholesterol-lowering therapies for the treatment of atherosclerosis.

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SUPPORTING INFORMATION AVAILABLE

Figures S1−S4 as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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