BIOCHEMISTRY
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Article

Biochemistry **2009,** *48,* 4423–4430 4423
DOI:10.1021/bi9001908

Functional Reconstitution of Human ABCC3 into Proteoliposomes Reveals a Transport Mechanism with Positive Cooperativity

Britta Zehnpfennig,*,‡ Ina L. Urbatsch,§ and Hans-Joachim Galla‡

‡*Institute of Biochemistry, University of Münster, 48149 Münster, Germany, and*
§*Department of Cell Biology and Biochemistry, Texas Tech University Health Science Center, Lubbock, Texas 79430*

Received February 5, 2009; Revised Manuscript Received March 18, 2009

ABSTRACT: ABCC3 (MRP3) is a member of the family of multidrug resistance-associated proteins (MRP), which belong to the largest family of membrane transport proteins, namely, the ATP binding cassette (ABC) transporters. Members of this family contribute to the excretion of several organic anions from cells and play a critical role in conferring resistance against drugs used in the treatment of cancer. The overexpression of ABCC3 in the yeast *Pichia pastoris* and its subsequent purification made possible the study of substrate-dependent ATPase activity [Chloupkova, M., et al. (2007) *Biochemistry 46*, 7992−8003]. Here we describe the successful reconstitution of purified ABCC3 in proteoliposomes and ABCC3-dependent uptake of the anticancer drug methotrexate (MTX), as well as the physiological substrate leukotriene C_4 (LTC_4). Our results show specific transport in a cell-free environment and in the absence of other proteins, revealing positive allosteric cooperativity for ABCC3-mediated substrate translocation. The ABCC3-mediated transport of MTX indicates a Hill coefficient of 2.3 ± 1.7, a maximum transport rate (V_{max}) of $> 2\ \mu$mol min^{-1} mg^{-1}, and a K_M in the millimolar range, whereas the translocation of LTC_4 into proteoliposomes displayed a Hill coefficient of 2.3 ± 0.5 with a maximum transport rate of 4.7 ± 0.8 nmol min^{-1} mg^{-1}, and a K_M in the micromolar range (1.7 ± 0.3 μM). The transport of both substrates, MTX and LTC_4, was inhibited by etoposide, confirming a higher affinity of ABCC3 for LTC_4 than for MTX. The technical advances described in this report represent the basis for the extended and detailed kinetic characterization of ABCC3 with a wide range of implications for the investigation of other human ABC transporters.

ATP binding cassette (ABC)[1] transporters belong to a superfamily of transmembrane proteins, which transport a variety of substrates across intra- and extracellular membranes (*1*). Some of these carrier proteins are responsible for the efflux of lipids, bile salts, and metabolites using the energy derived from ATP hydrolysis. Others are able to transport a variety of structurally diverse xenobiotics out of mammalian cells, thereby protecting tumor cells against medical drugs and preventing effective cancer treatment (*2*). This phenomenon is called multidrug resistance (MDR).

*To whom correspondence should be addressed: Institute of Biochemistry, WWU Münster, Wilhelm-Klemm-Str. 2, 48149 Münster, Germany. Phone: (0049-251) 833-3039. Fax: (0049-251) 833-3206. E-mail: b.zehnpfennig@uni-muenster.de.

[1]Abbreviations: ABC, ATP binding cassette; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BSA, bovine serum albumin; DDM, *n*-dodecyl β-D-maltoside; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; $E_2$17βG, estradiol 17β-D-glucuronide; LTC_4, leukotriene C_4; LUVs, large unilamellar vesicles; M3G, morphine 3-glucuronide; MDR, multidrug resistance; MLVs, multilamellar vesicles; MRP, multidrug resistance-associated protein; MTX, methotrexate; NBDs, nucleotide binding domains; PC, phosphatidylcholine; TMDs, transmembrane domains.

The ABCC subfamily of ABC transporters consists of nine members in humans (ABCC1−6 and ABCC10−12), which are also called MRPs [multidrug resistance-associated proteins (MRP1−9)], because of their ability to confer MDR to anticancer drugs in various cells. ABCC1, ABCC2, ABCC3, and ABCC4 are primarily responsible for the efflux of the anticancer drug methotrexate (MTX), a cytostatic compound employed in the therapeutic treatment of acute lymphoblastic leukemia, osteosarcoma, and the Non-Hodgkin lymphoma (*3*,*4*). Furthermore, they translocate the glutathione conjugate leukotriene C_4 (LTC_4) across membranes (*5*), an eicosanoid that is an important chemotaxin mediating inflammatory response. ABCC3 (MRP3) was found in the liver, kidney, intestine, adrenals, pancreas, gallbladder, and spleen (*6*). In the liver, expression of ABCC3 is highly inducible, and it is upregulated in the absence of functional ABCC2 and during cholestasis (*7*). Substrate-dependent induction has also been observed by several bile salts in an enterocytic cell line (*8*). Besides the xenobiotic drug MTX, ABCC3 has been shown to transport multiple organic substrates like glucuronidated compounds, e.g., estradiol 17β-D-glucuronide

Published on Web 3/31/2009

($E_2$17βG) (*9*) and morphine 3-glucuronide (M3G) (*10*), as well as bile salts (*11*).

To date, many approaches to exploring the specificities for substrates and inhibitors of different ABC transporters, using cell culture systems (*12*−*14*) or membrane vesicles from cultured cells (*5*,*6*,*12*,*15*,*16*), have been reported. However, the specificities for the transported substrates as well as the details of the transport mechanisms have been difficult to segregate due to the presence of other intrinsic transport proteins and fluctuations in the transfection efficiencies. Some successful attempts to functionally reconstitute purified ABC transporters have been performed in the case of bacterial transport proteins (*17*,*18*); however, the low abundance of mammalian transporters in their natural tissue or in transfected mammalian cell lines makes the production of required amounts of protein a difficult task (*19*,*20*).

Recently, heterologous expression of full-size human ABCC3 in the yeast *Pichia pastoris*, purification via affinity chromatography, and characterization of the protein employing specific ATPase activity measurements were reported (*21*). In accordance with in vivo and in vitro studies, the isolated transporter exhibited enhanced activity with respect to stimulation by several bile salts and glucuronated conjugates. In contrast, no significant ability to stimulate the ATPase activity of ABCC3 was observed for a variety of anticancer drugs like MTX, vincristine, and etoposide. Whereas differences in the ATPase activity of detergent-solubilized ABCC3 provide valuable information regarding substrate and inhibitor characteristics, they fail to give detailed insights into the vectorial translocation of substrates across the membrane. In this study, we introduce a systematic approach for the successful functional reconstitution of isolated human ABCC3 into artificial membrane systems and the detailed kinetic characterization of ABCC3-mediated transport of MTX and LTC_4 into proteoliposomes.

MATERIALS AND METHODS

Materials. CHAPS, Triton X-100, and asolectin were from Fluka (Seelze, Germany). *n*-Dodecyl β-D-maltoside (DDM) was obtained from AppliChem (Darmstadt, Gemany). Liver lipids were from Avanti (Alabaster, AL); brain total lipids, brain PC, and egg PC were purchased from Sigma (Munich, Germany). SDR HyperD solvent-detergent removal resin was from Pall (Dreieich, Germany). Unlabeled leukotriene C_4 (LTC_4) was obtained from Biozol (Eching, Germany); etoposide and methotrexate (MTX) were from Sigma. [^{3}H]LTC_4 and [^{3}H]MTX were purchased from Hartmann Analytic (Braunschweig, Germany). Scintillation liquid (Filtersafe) was from Zinsser Analytic (Frankfurt, Germany). All standard chemicals for buffers and media were from Sigma or AppliChem.

Growth of Fermenter Cultures and Preparation of Microsomes. The ABCC3-overexpressing yeast *P. pastoris* (*21*) was grown in fermenter cultures as described previously (*22*). Cells from 16 L fermenter cultures (~2.5 kg of wet cell mass) were harvested using a flow centrifuge (CEPA, Carl Padberg Zentrifugenbau GmbH, Lahr, Germany) and stored at 70 °C. Preparation of microsomal membranes was achieved by cell disruption in a Bead Beater (Hamilton Beach) as described previously (*23*) after resuspension of 80 g of wet cells in 120 mL of homogenization buffer [0.33 M sucrose, 0.3 M Tris, 1 mM EGTA, 1 mM EDTA, and 100 mM 6-aminohexanoic acid (pH 7.4)].

Purification of ABCC3. For purification of recombinant ABCC3, an established protocol with slight modifications (change in detergent concentration and use of 2-mercaptoethanol as reducing agent) was used (*23*). Microsomes were solubilized in 0.5% (w/v) DDM, and 0.1% (w/v) DDM was used during the chromatography on Ni-NTA and calmodulin resin for stabilization of the protein. Purified ABCC3 was concentrated ≤10-fold by ultrafiltration under nitrogen in an Amicon stirred cell (Milipore) using a PLTK ultrafiltration disk with a nominal cutoff of 30 kDa. Concentrations of the purified ABCC3 were determined by UV spectroscopy using a predicted molar extinction coefficient ε_{mol} of 230020 L mol^{-1} cm^{-1}. Functionality of the isolated ABC transporter was ensured by ATPase assays in solution as described previously (*21*).

Preparation of Large Unilamellar Vesicles (LUVs). For preparation of thin lipid films, dry lipids were dissolved in chloroform, dried slowly in a water bath under a stream of argon, and pumped under vacuum for 2 h at 40 °C. Lipid films were resuspended in reconstitution buffer [20 mM Tris, 100 mM NaCl, and 1 mM $MgCl_2$ (pH 7.4)] by being mixed after incubation at 40 °C for 30 min, resulting in final concentrations of multilamellar vesicles (MLVs) of up to 40 mM. LUVs were prepared by membrane extrusion of MLVs at 40 °C in a Liposofast (Avestin), using a polycarbonate membrane with a pore size of 200 nm.

Determination of the Point of Detergent Solubilization (R_{sol}) and Saturation (R_{sat}) of LUVs. For the determination of the point of saturation (R_{sat}) and the point of solubilization (R_{sol}), prepared LUVs at concentrations of 1, 5, and 10 mM were incubated with different detergents at various ratios at 23 °C for 10 min (Triton X-100) or 1 h (CHAPS and DDM), and then an absorption spectrum was recorded between 220 and 550 nm. The extinction at a certain wavelength outside the absorption maximum (320 nm for CHAPS, 350 nm for Triton X-100, and 300 nm for DDM) was plotted against the molar ratio of detergent to lipid for determination of the solubilization grade. R_{sat} and R_{sol} are consistent with the x values at the end of the left horizontal asymptote and the beginning of the right asymptote, respectively.

Preparation of Proteoliposomes. LUVs were destabilized with an amount of detergent close to R_{sat}, never exceeding R_{sol}, using a lipid concentration between 7 and 28 mM. After incubation for 1 h, purified ABCC3 and 1 mM DTT were added, matching a lipid-to-protein (w/w) ratio of 200/1, 100/1, or 50/1. The mixture was incubated at room temperature for 10 min. Detergent was removed by two sequential additions of SDR HyperD (25 mg of total/mg of lipid), each followed by incubation on ice for 30 min with gentle agitation. The resin was centrifuged for 1 min at 6500 × *g* and 4 °C; the supernatant was collected, and the resin was washed twice with reconstitution buffer. Proteoliposomes were harvested

by ultracentrifugation of the combined supernatants in a TLA120.1 rotor (Beckman) at 200000×*g* and 4 °C for 45 min. Pellets were resuspended in reconstitution buffer, yielding final lipid concentrations between 30 and 130 mM, and were frozen in liquid nitrogen and stored at −70 °C until use. Vesicle recovery and protein recovery were determined by phosphorus analysis of the harvested liposomes and BCA assays of the reconstituted protein.

ATPase Assays of Proteoliposomes. Frozen liposomes were carefully thawed on ice and extruded five times through a polycarbonate membrane (200 nm pore size) for separation. To prevent oxidation of the protein, 2.5 mM DTT was added. For ATPase assays with resolubilized ABCC3, 10 mM DDM was added to the liposomes. The suspension was given to the same volume of 20 mM MgATP, resulting in concentrations of 10 mM ATP and 11 mM Mg^{2+}, to start the reaction. The ATPase assays were conducted as described previously (*23*), but with the additional presence of 2 mM DDM in the ice-cold 20 mM H_2SO_4 to avoid scattering by the liposomes in the absorption assay. After each experiment, protein and lipid concentrations of the proteoliposomes were determined.

Transport of Substrates into Proteoliposomes. The ATP-dependent uptake of radiolabled substrates and inhibition was assessed by scintillation assays. Frozen liposomes were carefully thawed on ice and separated by being pushed five times through a polycarbonate membrane (200 nm pore size). DTT (2.5 mM) was added to prevent unintentional oxidation of the protein.

Then 220 μL of 2× transport buffer was prepared by mixing reconstitution buffer with 20 mM $MgCl_2$ and 20 mM ATP, followed by addition of a particular amount of substrate and, in the case of inhibition experiments, the inhibitor etoposide. Tested substrates MTX and LTC_4 were added from stocks in DMSO or 95% EtOH, leading to concentrations of not more than 2% DMSO or 3.5% EtOH in the assays. The radioactive ^{3}H-labeled component was added, yielding a total radioactivity of up to 0.5 μCi per sample. The assay was started by addition of 220 μL of the proteoliposome suspension and conducted at 37 °C. Aliquots were taken and filtered immediately under vacuum by pouring the suspension on top of a prewetted nitrocellulose membrane with a pore size of 0.2 μm. The filter was washed immediately four times with ice-cold reconstitution buffer. Dried filters were incubated in 2 mL of scintillation liquid for complete solubilization of the liposomes, and radioactivity was determined by scintillation counting. The amount of substrate translocated into the proteoliposomes was calculated using known amounts of ^{3}H-labeled substrate for calibration. In control experiments, ATP was replaced with AMP. After each experiment, the concentration of ABCC3 in the sample was measured. For the evaluation of kinetic data, initial transport rates were determined and plotted against the substrate concentrations. Data were fitted using the Michaelis−Menten equation or the Hill equation.

Protein and Lipid Assays. The amount of ABCC3 reconstituted in proteoliposomes was determined with the BCA protein assay in the presence of 2% (w/v) SDS to prevent interference with lipids, using BSA as a standard. The lipid concentrations for determination of the vesicle recovery were determined by assaying total phosphorus. Samples were mixed with 200 μL of perchloric acid (70%) and heated to 200 °C for 1 h. After the sample had cooled, 500 μL of 22 mM $(NH_4)_2MoO_4$ in 12.5% $HClO_4$ and 500 μL of 100 mM ascorbic acid were added to the samples. The mixture was boiled for 5 min and absorbance quantified at 810 nm. A calibration curve was generated by the same treatment of increasing amounts of a NaH_2PO_4 solution.

RESULTS

Reconstitution of ABCC3 into Proteoliposomes. It has previously been shown that the key to successful reconstitution of membrane proteins in functional form is the direct incorporation of the protein into preformed unilamellar liposomes (LUVs) destabilized with detergent to a certain extent of solubilization (*24*,*25*). Therefore, the molar detergent-to-lipid ratios yielding R_{sat} and R_{sol} were determined for each detergent using LUVs from asolectin lipids. In the case of CHAPS, the molar ratios resulting in R_{sat} and R_{sol} were 0.2 and 3.0, respectively. For Triton X-100, R_{sat} and R_{sol} were achieved with detergent-to-lipid ratios of 0.5 and 1.5 and for DDM of 0.4 and 1.5, respectively. Four different parameters were optimized in the evaluation of the reconstitution process for ABCC3 into proteoliposomes: (i) the type of the detergent, (ii) the amount of detergent, (iii) the lipid-to-protein ratio, and (iv) the lipid composition. To test the reconstitution efficiencies, we determined the total phospholipid as well as the integration of ABCC3 into the liposomes. The functionality of the reconstituted protein was validated by assaying the ATPase activity of ABCC3 incorporated in the proteoliposomes.

Effects of the Detergent on the Reconstitution of ABCC3. +We found that the type of detergent used for destabilization of phospholipid vesicles during preparation greatly influenced the yield of proteoliposomes (Figure 1A). The best recovery of protein (62 ± 2%) and lipids (79 ± 7%) in the proteoliposomes was achieved when the nonionic alkyl maltoside DDM was used for destabilization of LUVs, whereas the nonionic polyoxyethylene Triton X-100 led to a recovery of 55 ± 3% lipids and 49 ± 4% protein. When the zwitterionic detergent CHAPS was used for LUV destabilization, only 13 ± 2% of the initially employed lipids and < 10% of the protein could be recovered. In this case, we found that phospholipids remained dissolved in mixed detergent/lipid micelles, which were detected in the supernatant after ultracentrifugation (data not shown), likely due to insufficient binding of the detergent to the sorbent, thus preventing proteoliposome formation. The activity of reconstituted ABCC3 was determined by ATPase activity assays (Figure 1B). Proteoliposomes prepared with DDM showed a specific activity of 75 ± 4 nmol min^{-1} mg^{-1}, which was 2-fold higher than for those prepared in the presence of Triton X-100 (34 ± 3 nmol min^{-1} mg^{-1}). Proteoliposomes prepared with CHAPS were not examined due to the low reconstitution efficiency. Figure 1B reveals that the specific ATPase activity of resolubilized ABCC3 remained unaffected by

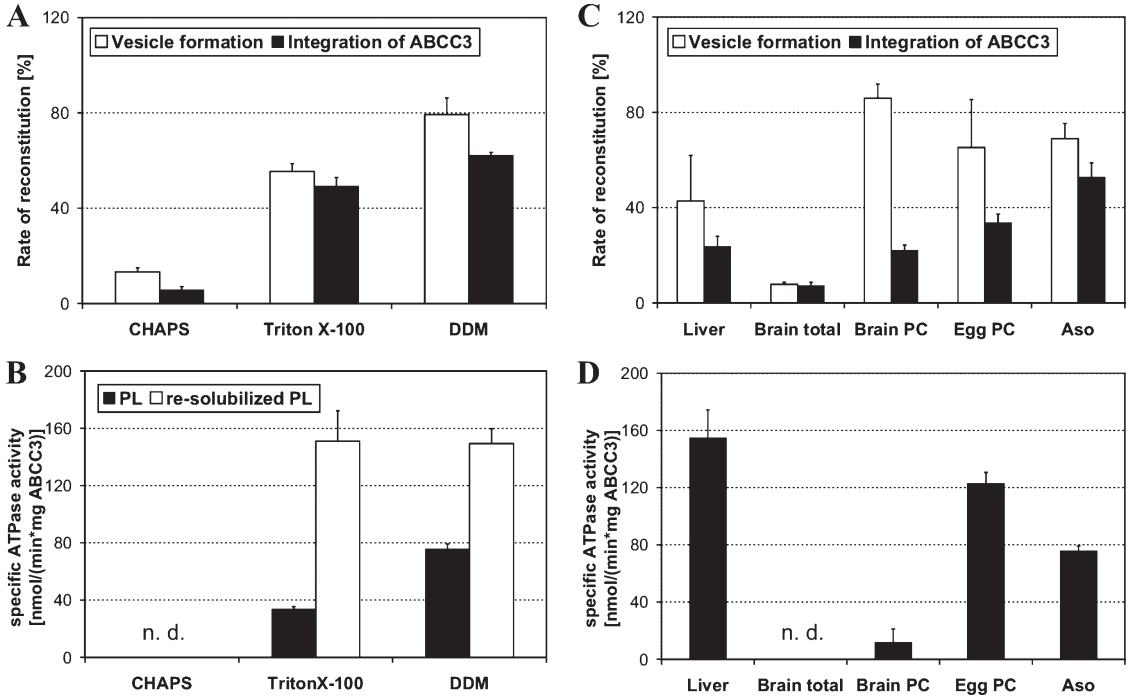

FIGURE 1: Influence of detergent and lipids on ABCC3 reconstitution in proteoliposomes. (A) The effect of the detergent on the recovery of lipids (white bars) and incorporated ABCC3 (black bars) was determined after destabilizing the preformed LUVs with the detergents CHAPS, Triton X-100, and DDM ($n \geq 3$). (B) The specific ATPase activity of intact proteoliposomes (PL, black bars) and proteoliposomes resolubilized with DDM (resolubilized PL, white bars) was determined for proteoliposomes from panel A. (C) The effect of the lipid composition on lipid recovery (white bars) and incorporation of ABCC3 (black bars) was determined for preparations with lipid extract from liver (Liver), total lipid extract from bovine brain (Brain total), brain phosphatidylcholine lipids (Brain PC), phosphatidylcholines from egg yolk (Egg PC), and soybean asolectin (Aso). (D) The specific ATPase activity of ABCC3-containing proteoliposomes was determined for proteoliposomes from panel C. The averages and standard errors of at least three independent experiments are shown.

the reconstitution procedure, regardless of the detergent used. On the basis of these ATPase assays, we assume that the lower activity was likely caused by the orientation of ABCC3 in the lipid bilayer with ~50% of the nucleotide binding domains (NBDs) facing outside the liposomes being available for ATP hydrolysis in the case of DDM and only ~25% being accessible in the case of Triton X-100. However, additional effects such as membrane fluidity may also affect the ATPase activity of the respective proteoliposome preparations. We also tested the effect of different DDM-to-lipid ratios on the reconstitution efficiency and activity of ABCC3. No significant differences in the vesicle recovery or in the quantity of integrated ABCC3 were observed with variation in the molar detergent-to-lipid ratios from 0.5 to 1.0 (data not shown). However, substantial differences in the ATPase activities were detected with the highest specific activity of reconstituted ABCC3 seen at a DDM-to-lipid ratio of 0.8 [75 ± 4 nmol min^{-1} mg^{-1}]. Molar ratios of 0.5 DDM to lipid (55 ± 4 nmol min^{-1} mg^{-1}) and 1.0 DDM to lipid (43 ± 5 nmol min^{-1} mg^{-1}) revealed significantly lower ATPase activities. Therefore, a DDM-to-lipid ratio of 0.8 was chosen for all subsequent preparations of proteoliposomes. Furthermore, variation of the lipid-to-protein ratios during the preparation of proteoliposomes from 200/1 to 50/1 (w/w) had no significant effect on the reconstitution efficiency of ABCC3, as well as on the specific ATPase activity (data not shown). Therefore, we used a lipid-to-protein ratio of 100/1 or 50/1 for all subsequent assays.

Effects of Different Lipids on the Reconstitution of ABCC3. Different transport proteins are found in different lipid environments, e.g., various cell types, organelles, or opposite membranes of polarized cells. Therefore, we tested different lipids for the optimization of the reconstitution system, using a lipid-to-protein ratio of 50/1 (w/w). ABCC3 is expressed in the liver (*26*), and enhanced ATPase activity in the presence of liver lipids has been shown for ABCC3 in solution (*21*). Small amounts of ABCC3 were also detected in brain (reviewed in ref *27*). Therefore, we selected liver and brain lipids (bovine, total lipid extracts), which are rich in PC lipids, as well as brain phosphatidylcholine (PC) lipids, purified PC lipids from egg yolk (>99% pure), and asolectin from soybean. Large differences in the reconstitution efficiencies were observed for the different lipid systems (Figure 1C). Liposomes from asolectin exhibited the highest level of insertion of ABCC3 together with adequate vesicle recovery. Although brain PC also yielded high lipid recovery in the prepared proteoliposomes, only poor incorporation of ABCC3 was achieved. Proteoliposomes from liver or egg PC varied strongly in vesicle formation (large error bars) and gave inadequate insertion of ABCC3 compared to asolectin. Reconstitution into brain total lipids led to insufficient vesicle formation with small amounts of protein. The highest specific ATPase activity of reconstituted ABCC3 was observed in liver lipids (155 ± 20 nmol min^{-1} mg^{-1}) and egg PC (123 ± 8 nmol min^{-1} mg^{-1}) (Figure 1D). In proteoliposomes from asolectin, the specific ATPase activity was 76 ± 3 nmol min^{-1} mg^{-1}.

ABCC3 reconstituted in brain PC exhibited almost no activity (12 ± 9 nmol min^{-1} mg^{-1}). Because of their highest yields in terms of vesicle formation and insertion of ABCC3 as well as significant and reproducible rates of ATP hydrolysis, the asolectin system was used in all further investigations.

Effect of the Potential Substrate MTX on ATPase Activity. Since stimulation of the ATPase activity by various potential substrates has previously been shown for purified ABCC3 in solution (*21*), this influence of certain substrates on specific activity remained to be proven for our model system of reconstituted ABCC3 in proteoliposomes. We selected the cytostatic methotrexate (MTX) as a representative substrate of ABCC3 (reviewed in ref *27*) to determine the effect of substrate concentration on the ATPase activity of the asolectin-reconstituted protein.

The specific ATPase activity of proteoliposomes prepared at a molar detergent-to-lipid ratio of 0.8 and with a lipid-to-protein ratio of 100/1 (w/w) was determined (Figure 2A). In the absence of MTX, the specific activity was 61 ± 5 nmol min^{-1} mg^{-1}. In the presence of MTX, the ATPase activity increased with MTX concentrations of up to 2 mM (265 ± 27 nmol min^{-1} mg^{-1}); higher concentrations of MTX were not soluble in the aqueous medium.

Transport of MTX into Proteoliposomes. To determine the kinetic parameters of substrate transport, we performed uptake assays with ABCC3-containing proteoliposomes prepared under conditions identical to those described for Figure 2A. No significant uptake of MTX was detected when the assay was performed in the presence of AMP, reconfirming the energy requirement of ABCC3-mediated transport in this system (Figure 2B). In the presence of 10 mM ATP, the uptake of 1.75 mM MTX exhibited an initial transport rate of 777 ± 58 nmol min^{-1} mg^{-1}. To determine the affinity of ABCC3 for MTX in our transport assay, the initial rates of transport were determined at different concentrations of MTX (Figure 2C). In previous studies using membrane vesicles from transfected mammalian cells (*5*,*15*), ABCC3 was shown to exhibit Michaelis−Menten kinetics, revealing MTX transport by ABCC3 with low affinity and high capacity. However, evaluation of data using the Michaelis−Menten equation did not provide an adequate description of the data received from ABCC3-mediated uptake into proteolipsomes. Therefore, the data were fitted to the Hill equation (solid line, Figure 2C). Note that due to the limited solubility of MTX, uptake in the presence of concentrations of >2 mM could not be determined. Hence, we were unable to obtain reliable kinetic constants but estimated a Hill coefficient of 2.3 ± 1.7, indicating very strong cooperativity for MTX transport mediated by ABCC3. The maximum transport rate (V_{max}) was >2 μmol min^{-1} mg^{-1} with a K_M in the millimolar range. These transport rates of purified ABCC3 reconstituted in proteoliposomes are similar to the rates derived from ABCC3-mediated uptake into membrane vesicles from transfected cell lines (V_{max} = 20.2 ± 5.9 pmol min^{-1} mg^{-1}) (*5*), taking into consideration the total amount of membrane protein with an extrapolated ABCC3 content of ~0.5%.

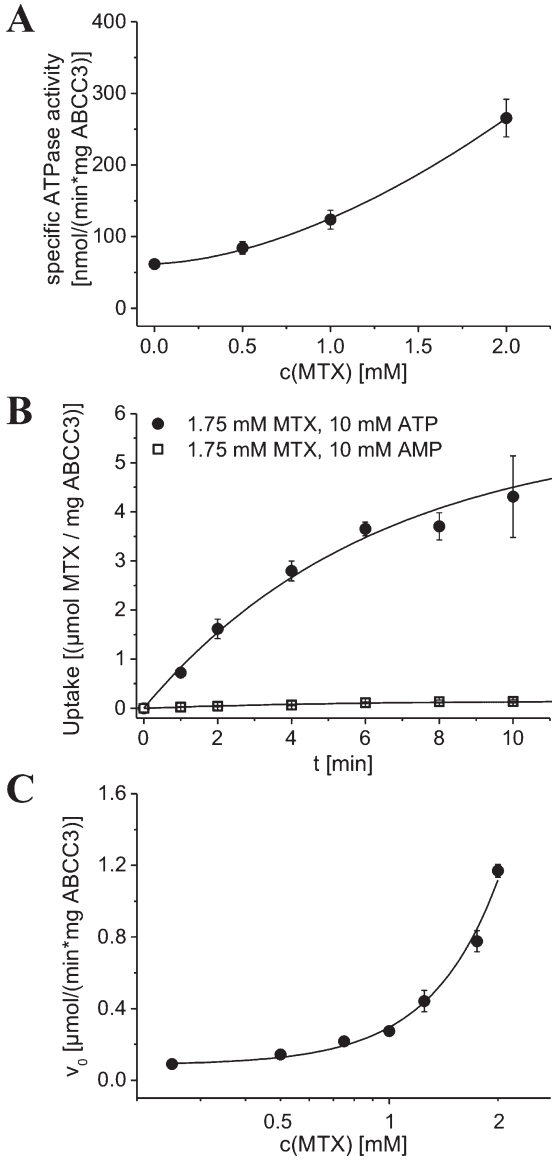

FIGURE 2: Kinetics of MTX uptake. (A) ATPase activity of ABCC3 reconstituted in proteoliposomes in response to different concentrations of MTX. Data were fitted to the Hill equation with a Hill coefficient of 1.7 ± 0.3. (B) Time course of ABCC3-mediated uptake of MTX into proteoliposomes in the presence of 1.75 mM MTX and either 10 mM ATP (●) or AMP (□). (C) Initial transport rates of MTX uptake by ABCC3 plotted as a function of substrate concentration. The solid line is a fit to the Hill equation with a Hill coefficient of 2.3 ± 1.7. The averages and standard errors of at least three independent experiments are shown.

Transport of LTC_4 into Proteoliposomes. As a representative of physiological substrates for active transport mediated by ABCC3, we chose the glutathione conjugate leukotriene C_4 (LTC_4) for further uptake studies. In contrast to MTX, this compound was reported to be translocated by ABCC3 with moderate affinity but low capacity (*5*). We found that in the presence of AMP the uptake activity of ABCC3 was very low (v = 0.36 ± 0.14 nmol min^{-1} mg^{-1}), which may be attributed to passive diffusion of LTC_4 into the proteoliposomes. In the presence of ATP, uptake of LTC_4 exhibited an initial rate of transport of 3.7 ± 0.2 nmol min^{-1} mg^{-1} (Figure 3A). The initial rates of active transport were determined as a function of LTC_4 concentration (Figure 3B). Uptake was

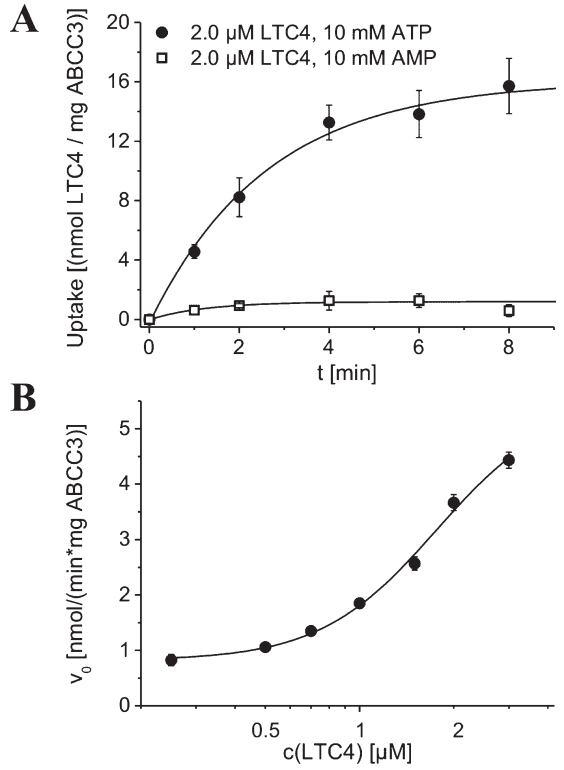

FIGURE 3: Kinetics of LTC_4 uptake. (A) Time course of ABCC3-mediated uptake of LTC_4 into proteoliposomes in the presence of 2 μM LTC_4 and either 10 mM ATP (●) or AMP (□). (B) Initial transport rates of LTC_4 uptake by ABCC3 plotted as a function of substrate concentration. Data were fitted to the Hill equation (solid line) with a Hill coefficient of 2.3 ± 0.5. The averages and standard errors of at least three independent experiments are shown.

saturable with a K_M of 1.7 ± 0.3 μM and a V_{max} of 4.7 ± 0.8 nmol min^{-1} mg^{-1}. We determined a Hill coefficient of 2.3 ± 0.5 for LTC_4 transport, suggesting positive cooperativity of this substrate, too. The relatively low K_M and low V_{max} support previous results in membrane vesicles of ABCC3 being a transport system with moderate affinity and low capacity for LTC_4 when compared to the MTX transport characteristics (high K_M and high V_{max}) (*5*).

Inhibition of Transport by Etoposide. Finally, reconstituted ABCC3 in proteoliposomes was employed for quantification of transport inhibition by a common inhibitor for ABC transporters, the antagonistic glycoside derivative etoposide (*15*). Uptake assays were performed as described above, but with etoposide concentrations from 0 to 500 μM. In these experiments, the concentrations of MTX and LTC_4 were 1 mM and 1 μM, respectively. The initial transport rates in the presence of etoposide were compared to the ABCC3-mediated transport without inhibitor (Table 1). For both substrates, transport rates decreased reciprocally to the concentration of etoposide. In the case of MTX, active transport was inhibited completely by addition of 500 μM etoposide with an IC_{50} of ~145 μM (7-fold excess of substrate), indicating a higher affinity of ABCC3 for etoposide than for MTX. In the presence of up to 500 μM etoposide, the active transport of LTC_4 was attenuated significantly, but complete inhibition was not achieved; 50% inhibition (IC_{50}) was observed at ~160 μM (160-fold excess of inhibitor), indicating an excessively higher affinity of ABCC3 for LTC_4 than for etoposide.

Table 1: Inhibition of ATP-Dependent Uptake by Different Concentrations of Etoposide

[etoposide] (μM)	ATP-dependent transport[a] of 1 mM MTX	ATP-dependent transport [a] of 1 μM LTC_4
0	100	100
50	82 ± 6	84 ± 5
100	65 ± 4	61 ± 8
200	35 ± 3	44 ± 9
500	< 1	28 ± 6

[a] Expressed as the percentage of the control in the absence of inhibitor.

DISCUSSION

We describe the first successful incorporation of purified ABCC3 into proteoliposomes and the detailed characterization of ABCC3-mediated transport of the anticancer drug MTX and the chemotaxin LTC_4 across the lipid bilayer. To obtain reliable kinetic data, we optimized the reconstitution protocols, ensuring stability of the protein and maintenance of functionality during the reconstitution procedure. As a consequence, we were able to characterize human ABCC3 derived from the yeast *P. pastoris* in an artificial membrane system by means of ATPase activity and substrate transport.

We observed significant differences in the ATPase activity of the reconstituted protein in response to different detergents used for the reconstitution procedure. Using DDM for the solubilization of the preformed LUVs resulted in the highest specific ATPase activity of ABCC3. Significantly lower ATPase activity was observed with intact proteoliposomes prepared in the presence of Triton X-100. Since the ATPase activities of ABCC3 originating from resolubilized proteoliposomes prepared with either DDM or Triton X-100 were indistinguishable (Figure 1B), we conclude that Triton X-100 predominantly mediated the incorporation of the transporter with the NBDs facing inside the proteoliposomes and inaccessible to the ATP medium rather than causing denaturation or inactivation of ABCC3. This notion is supported by the fact that Triton X-100-mediated protein incorporation in a preferred orientation has been reported for several membrane proteins (*28*), whereas the reconstitution with DDM has led to random orientations (*29*). In addition to the detergent, we found that the lipid environment greatly influenced the reconstitution efficiency and the activity of ABCC3 in proteoliposomes. Whereas the highest ATPase activity was determined in lipid extracts derived from liver, lipids from brain proved to be inferior for the functional ABCC3 reconstitution due to poor lipid recovery in the liposomes. This observation (Figure 2D) is in accordance with the relatively high level of ABCC3 expression in liver (*26*). However, due to the variations in vesicle formation and inadequate yields of incorporated ABCC3 in the proteoliposomes made of liver lipids, as well as those from egg PC (Figure 2C), we decided to perform our kinetic studies in proteoliposomes made of asolectin.

Some representatives of the ABC transporter family are involved in a phenomenon called multidrug resistance, due to their ability to translocate a broad spectrum of anticancer drugs across cellular membranes. Binding of substrates to the transmembrane domains (TMDs) of the transporter enhances ATPase hydrolysis (reviewed in ref *30*) and leads to an increasing level of substrate translocation in the case of drug exposure. This effect was observed for ABCB1, leading to a 10-fold increase in its activity (*22*), whereas members of the ABCC family did only exhibit increased stimulation up to 1.5-fold in the case of ABCC1 (*31*) and 2−3.5-fold for ABCC2 (*20*). In contrast, no significant stimulation of ATPase activity was observed for purified ABCC3 in solution in the presence of various anticancer drugs, including MTX, contrary to significantly enhanced activity in ATP hydrolysis in the presence of bile salts and glucuronides (*21*). However, in transfected cell lines and a diversity of ABCC3-expressing human lung cancer cell lines, ABCC3-related resistance to drugs like MTX, etoposide, teniposide, and vincristine has been reported (*32*−*34*). In our vesicular system with reconstituted ABCC3, the specific ATPase activity was observed to increase significantly at high concentrations of MTX (2-fold with 1 mM MTX and 5-fold in the presence of 2 mM MTX), suggesting that drugs and lipids interact and that removal of detergent is necessary to mimic the physiological environment of the transport protein. Thus, our system with functional reconstituted ABCC3 in proteoliposomes represents an appropriate model for the specific investigation of the activity of isolated ABC transporters.

We employed our model system for the investigation of drug transport, using the antimetabolite MTX in vesicular transport assays. Plotting the initial rates of MTX transport indicated concentration-dependent ABCC3-mediated uptake; however, "simple" Michaelis−Menten kinetics could not be applied for the ABCC3-mediated MTX transport. While our data confirm the assumption that MTX is transported by ABCC3 with low affinity, but high capacity, in membrane vesicles from transfected cell lines (*5*), we observed positive cooperative allosteric effects for translocation of MTX by ABCC3, displaying a Hill coefficient of > 1. These allosteric effects had been described previously for other MRPs, like homotropic allostery in the case of ABCC4 transporting urate or cGMP (*12*). Heterotropic allosteric effects had been observed in ABCC1-mediated transport of various substrates enhanced by GSH and for translocation of $E_2 17\beta G$ by ABCC2 (*35*). For Pgp (ABCB1), positive cooperativity of at least two substrate binding sites had been found (reviewed in ref *2*). In contrast, no cooperativity had been described for ABCC3-mediated uptake of anticancer drugs. Presumably, this might be attributed to the typical use of membrane vesicles prepared from transfected cell lines for transport investigations (*5*,*15*). These membrane fragments contain a variety of proteins, including ABC transporters, wherefore determined effects must not be attributed to ABCC3 only. The Hill coefficient for transport of MTX by ABCC3 suggests that ABCC3 possesses multiple allosteric substrate binding sites, which provides an advantage by mediating drug resistance to the cell, where local concentrations in direct cellular ambience may be higher than the average blood concentration. The substrate concentrations in the uptake assays are in the range of plasma levels during high-dose MTX therapy in cancer treatment. For example, in treatment of children or adults with osteosarcoma, most patients have serum concentrations in the millimolar range ($\geq 1000\ \mu M$) after intravenous application of 12 g/m^2 of body surface area over 4 h (*3*). Our results are consistent with previous studies, where ABCC3 was observed to confer resistance during short-term exposure to high MTX concentrations (*32*). This reveals the important involvement of ABCC3 in the protection of human tissues and organs, as well as in the clinical phenomenon of multidrug resistance.

ABCC3 is also known to export some glucuronides and glutathione conjugates. One of these physiological substrates is leukotriene C_4 (LTC_4), which is generated in leukocytes from hydrolysis of phospholipids by phospholipase A_2 or in hepatocytes and endothelial cells by LTC_4 synthase. Therefore, we employed this compound as a representative of substrates in human metabolism for further investigations of ABCC3 transport properties. In congruence to our preceding studies with the anticancer drug MTX, positive cooperative allostery appears, leading to a Hill coefficient of ~ 2.

We showed that ABCC3 not only mediates translocation of physiological compounds but also is involved in maintaining resistance to cytotoxic drugs, e.g., MTX. Because cellular resistance to anticancer drugs mediated by ABC transporters is a major problem in the treatment of cancer patients, specific inhibition of active drug transport is a significant issue in cancer research. Another anticancer drug described in literature is the epipodophyllotoxin etoposide. This antagonistic glycoside derivative was revealed to be an effective inhibitor for ATP-dependent uptake of MTX in membrane vesicles (*15*). With regard to these findings, we used this common inhibitor for ABCC3-mediated uptake to monitor inhibition of substrate translocation in our model system. However, the level of uptake of the antimetabolite MTX at a given concentration of 1 mM was decreased depending on etoposide concentrations in the assay. Complete suppression could be attained by addition of 500 μM inhibitor, revealing an affinity of etoposide for ABCC3 higher than the affinity observed for MTX. In contrast, the rate of ABCC3-mediated uptake of LTC_4 (1 μM) into proteoliposomes was reduced by etoposide, indeed, but even in the presence of 500 μM inhibitor, we did not observe complete suppression of active transport, though the concentration of etoposide was 500-fold higher than the concentration of the substrate. Therefore, we assume the epipodophyllotoxin etoposide had a lower affinity for ABCC3 than the glutathione conjugate LTC_4.

In conclusion, data generated from our reconstitution system of ABCC3 in proteoliposomes have implications for the involvement of ABCC3 in drug resistance, as well as in physiological procedures like export of glutathione conjugates. Further, we developed an adequate model for systematic characterization of substrate specificities, which can be applied to the investigation of other human ABC transporters.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. Alexander Steinbüchel (Westfälische Wilhelms-Universität, Münster, Germany) for enabling the fermentation of the ABCC3-overexpressing yeast *P. pastoris* and Dr. Matthias Quick (Columbia University, New York, NY) for the critical reading of the manuscript.

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