Biophysical Chemistry 171 (2013) 31–37

Contents lists available at SciVerse ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Substrate translocation and stimulated ATP hydrolysis of human ABC transporter MRP3 show positive cooperativity and are half-coupled

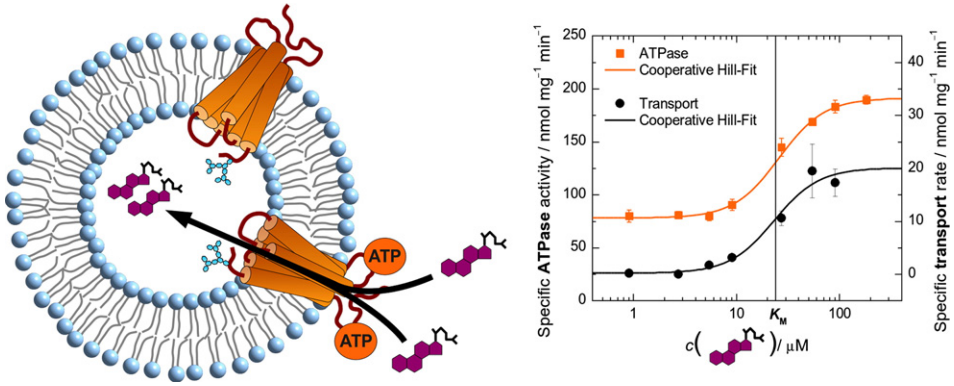
Patrick Seelheim [a,b], Adriane Wüllner [a], Hans-Joachim Galla [a,*]

[a] Institute for Biochemistry, University of Muenster, Wilhelm-Klemm-Str. 2, 48149 Muenster, Germany
[b] International Graduate School of Chemistry, University of Muenster, Wilhelm-Klemm-Str. 10, 48149 Muenster, Germany

HIGHLIGHTS

- Wild-type human MRP3 was reconstituted into large unilamellar vesicles.
- Reconstitution into proteoliposomes is crucial to get reliable kinetic parameters.
- Stimulation of ATPase and substrate translocation show positive cooperativity.
- Maximal stimulation of ATPase activity is substrate independent.
- ATPase and transport show identical K_M values and are half-coupled.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 August 2012
Received in revised form 2 October 2012
Accepted 3 October 2012
Available online 11 October 2012

Keywords:
ABC transporter
MRP3
Cooperativity
Enzyme kinetic
Proteoliposomes
Transport mechanism

ABSTRACT

ABC transporters are involved in countless processes from lipid excretion over cellular detoxification to multidrug resistance of cancer cells. The latter is especially conferred by the ABCC subfamily also called multidrug resistance-associated proteins (MRPs) that excrete a variety of amphipathics including anticancer drugs by ATP-dependent transport. As the mechanisms of substrate translocation and ATP hydrolysis are still unclear for MRPs, we investigated the kinetics of both processes with focus on cooperativity and coupling between ATPase activity and substrate transport using purified MRP3 in proteoliposomes. Although the ATP-dependent uptake of amphipathics and the hydrophilic 5(6)-carboxy-2′-7′-dichlorofluorescein (CDCF) into the lumen of proteoliposomes showed affinity constants similar to those reported for cell-based assays, the maximal uptake rates were up to 250 times higher. Moreover, all substrates showed cooperative interactions of two subunits. Upon stimulation with amphipathics, ATPase activity of MRP3 increased from 80 nmol/(mg min) to 180 nmol/(mg min) showing positive cooperativity with a Hill coefficient of 2. While Hill coefficient and maximal ATPase activity were found to be substrate independent, the affinity constants are characteristic for a given substrate and correspond to the value for transport. Therefore, cooperative interactions of the two nucleotide binding domains (NBDs) in MRP3 are mediated by substrate binding to the transmembrane domains (TMDs). In contrast to amphipathic substrates, CDCF did not

Abbreviations: CDCF, 5(6)-carboxy-2′,7′-dichlorofluorescein; DDM, *n*-dodecyl β-D-maltoside; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; $E_2$17βG, β-estradiol 17-(β-D-glucuronide); LTC4, leukotriene C4; MRP, multidrug resistance-associated protein; MTX, methotrexate; NBD, nucleotide binding domain; Pgp, P-glycoprotein; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TMD, transmembrane domain.

* Corresponding author. Tel.: +49 251 83 33201; fax: +49 251 83 33206.
E-mail address: gallah@uni-muenster.de (H.-J. Galla).

0301-4622/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bpc.2012.10.001

stimulate ATPase activity despite being transported in an ATP-dependent manner. This indicates that ATP hydrolysis and substrate translocation are half-coupled in MRP3 as CDCF shuttles on a basal TMD activity resulting from the basal ATPase activity.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

ABC transporters comprise the largest superfamily of transmembrane proteins ubiquitous in all living organisms and share a common functional entity of two highly conserved NBDs, where ATP hydrolysis takes place, and two highly variable TMDs, which perform substrate binding and translocation. In humans, 48 ABC transporters actively export a multitude of physiological and xenobiotic substrates including steroids, anticancer drugs and glucuronide metabolites across lipid membranes [1,2]. The MRP subfamily of ABC transporters is over-expressed in many tumors and possibly confers multidrug resistance against chemotherapeutic drugs to cancer cells [3]. Although for some eukaryotic ABC transporters like Pgp, a crystal structure [4] and elaborate models for the catalytic cycle are available [5,6], neither the precise biological function of MRPs, nor the mechanism of substrate translocation and ATP hydrolysis are known so far. One reason for that is the difficulty of expressing and purifying large transmembrane proteins so that present data rely mostly on plasma membrane vesicles from cells over-expressing MRPs for transport studies or MRPs solubilized in (mixed) micelles for investigation of ATPase activity [7]. These approaches suffer from several drawbacks: In cellular vesicle preparations, there are always other membrane proteins and transporters present so that the observed effects cannot be definitely attributed to the over-expressed MRP. For example, the ATP hydrolysis by MRPs cannot be quantified in plasma membrane vesicles because of numerous other highly active membranous ATPases. These effects might explain why substrate transport is usually reported to follow non-cooperative Michaelis–Menten kinetics [8], instead of cooperative Hill-type kinetics as expected from the proposed catalytic cycle [9] and observed in previous work using purified protein in proteoliposomes [10]. However, milligram quantities of purified human wild-type MRP3 are now available [11], and preliminary studies on reconstitution into proteoliposomes were very successful [10]. Hence, we investigated stimulated ATP hydrolysis and substrate translocation of MRP3 reconstituted in proteoliposomes under identical conditions to answer the question of cooperativity. Further, these data provided insight into the coupling between ATP hydrolysis and substrate translocation.

2. Materials and methods

2.1. Expression and purification of MRP3

Microsomal membranes were prepared from 2.5 L fermenter (Infors Minifors) cultures of a MRP3-expressing yeast *P. pastoris* [11] following established protocols [12]. MRP3 was solubilized in DDM and purified by affinity chromatography on Ni-NTA and calmodulin resins as described previously [10] substituting TES for Tris in all buffers [13].

2.2. Preparation of proteoliposomes

MRP3 was reconstituted into large unilamellar vesicles made from asolectin (20 mM lipid) at a lipid-to-protein ratio of 50/1 (w/w) corresponding to molar ratio of approximately 11,000/1 as described previously [10] using a modified reconstitution buffer (20 mM TES-NaOH, pH 7.4; 100 mM NaCl, 1 mM $MgCl_2$). Proteoliposomes were flash-frozen in liquid nitrogen and stored at −70 °C. Prior to use, they were thawed on ice, incubated with 1 mM DTT to prevent oxidation and extruded five times through a polycarbonate membrane (Avestin Liposofast, 200 nm pore size).

2.3. ATPase assay

ATPase activity was monitored photometrically via an enzyme cascade leading to oxidation of NADH [14]. Briefly, 100 μL ATPase cocktail (50 mM TES-NaOH, pH 7.4; 40 mM NaCl, 10 mM KCl, 12 mM $MgSO_4$, 10 mM Na_2ATP, 1 mM phosphoenol pyruvate, 0.28 mM NADH, 5 U/mL pyruvate kinase, 7 U/mL lactate dehydrogenase) prewarmed to 37 °C were injected into wells of a 96well plate containing 10 μL proteoliposomes. The absorbance decrease at 355 nm was monitored for 30 min in intervals of 30 s at 37 °C with a plate photometer (Berthold Mithras LB 940). Substrates for ATPase stimulation were added from stocks in DMSO, ethanol or reconstitution buffer to ATPase cocktail prior to injection leading to solvent concentrations not exceeding 2%.

Specific ATPase activity was calculated from the slope of the absorbance decrease using a calibration curve for NADH and corrected for only 50% of MRP3 being accessible for ATP from the liposomal exterior [10].

2.4. Uptake of ^{3}H-labeled substrates into proteoliposomes

The ATP-dependent translocation of radio-labeled substrates into the lumen of proteoliposomes was assessed by liquid scintillation as described previously [15]. 200 μL transport buffer (50 mM TES-NaOH, pH 7.4; 41 mM NaCl, 10 mM KCl, 12 mM $MgSO_4$, 10 mM Na_2ATP) containing the substrate (from stocks in DMSO or ethanol; final concentration of solvent not exceeding 2%) doped with up to 0.5 μCi of the ^{3}H-labeled compound was prewarmed to 37 °C, and the assay was started by addition of 20 μL proteoliposomes. Every minute, 20 μL aliquots from the assay mixture were vacuum-filtered through prewetted nitrocellulose membrane filters (Whatman Protran BA 79). Filters were immediately washed with 4 × 1 mL cold reconstitution buffer, dried and incubated with 4 mL scintillation liquid (Zinsser Filtersafe) for liquid scintillation counting (Beckman LS 6500). Transport buffer was substituted by reconstitution buffer without ATP in negative controls.

The amount of translocated substrate was determined using a calibration curve for the radio-labeled substrate, and the specific transport rate was calculated from the initial slope and corrected for only 50% of MRP3 being accessible from the liposomal exterior. Passive permeation was negligible in all cases.

2.5. Uptake of CDCF into proteoliposomes

The ATP-driven uptake of CDCF into the lumen of proteoliposomes was determined by fluorescence spectroscopy. 500 μL transport buffer containing CDCF (from stocks in reconstitution buffer) was prewarmed to 37 °C, and the assay was started by addition of 50 μL proteoliposomes. After 1, 2, 3, 5 and 7 min 100 μL aliquots from the assay mixture were diluted into 500 μL cold reconstitution buffer to stop substrate translocation. Proteoliposomes in the sample fractions were pelleted by ultracentrifugation (TLA 120.1 rotor, 257,000 rcf, 25 min, 4 °C), washed with 4 × 0.5 mL cold reconstitution buffer without disturbing the pellet and finally solubilized in 100 μL Triton X-100 (0.1% (w/v) in reconstitution buffer), shaking vigorously at room temperature. Fluorescence of 75 μL aliquots was quantified in a plate photometer using 485 nm excitation and 535 nm emission filters (Berthold Mithras LB 940). Transport buffer was substituted by reconstitution buffer without ATP in negative controls. For quantification of translocated CDCF, a calibration curve was used, and the specific transport rate was calculated from the initial slope and corrected for both passive permeation in the absence of ATP and only 50% of MRP3 being accessible from the liposomal exterior.

2.6. Data evaluation

The Hill equation (Eq. (1)), where v_0 and v_{max} are basal and maximal activity, [S] is the substrate concentration, K_M is the apparent dissociation constant indicating half-maximal activation and n is the Hill coefficient, was fitted to kinetic data for ATPase activity using OriginPro 8. For datasets with six or less data points the Hill coefficient was set to 0.5, 1, 2, 3 and 4, and the fit with lowest χ^2 was chosen. When fitting substrate transport, basal activity v_0 was fixed at 0.

$$v = v_0 + (v_{max} - v_0)\frac{[S]^n}{[S]^n + K_M^n} \quad (1)$$

2.7. Liposome integrity

Influence of bile acids and DMSO on membrane permeability of liposomes was checked by efflux of carboxyfluorescein [16].

Large unilamellar liposomes (5 mM lipid) were prepared by extrusion through a polycarbonate membrane (Avestin Liposofast, 200 nm pore size) in presence of carboxyfluorescein at a self-quenching concentration of 50 mM and were separated from free dye by gel filtration on Sephadex G-25 using reconstitution buffer as eluent. In a 96well plate 10 μL liposomes were injected into 100 μL reconstitution buffer prewarmed to 37 °C and containing the desired concentration of bile acid or DMSO. Fluorescence (Berthold Mithras LB 940; 485 nm excitation, 535 nm emission) intensity $I(t)$ was monitored over 30 min in intervals of 1 min prior to recording the terminal fluorescence I_∞ after injection of Triton X-100 (0.1% (w/v) final concentration). The relative efflux of carboxyfluorescein over time was calculated as $(I(t) - I_0)/(I_\infty - I_0)$.

2.8. Lipid and protein assays

Concentration of purified MRP3 was determined by UV absorbance at 280 nm using a calculated molar extinction coefficient of $\varepsilon = 230{,}020\ M^{-1}\ cm^{-1}$. Purity was routinely checked by SDS-PAGE and western blotting using $RGSH_4$ antibody (Qiagen #34650).

Protein concentration of samples containing lipids was determined with the BCA assay in the presence of 2% (w/v) SDS using BSA as standard.

Lipid concentration was assayed photometrically as inorganic phosphate after ashing with 70% perchloric acid as described previously [17].

3. Results

3.1. Real-time ATPase assay

Stimulated ATPase activity of purified ABC transporters is the most readily accessible parameter for identifying putative substrates and characterizing their affinities. Hence, a rapid and sensitive high-throughput assay for ATP hydrolysis is necessary. Detection of ATP hydrolysis monitored as NADH oxidation via a coupled enzyme assay using a 96well plate photometer equipped with automatic injectors provides kinetic data with 30 s time resolution for up to 96 samples simultaneously (Fig. 1A). After an initial equilibration phase of about 1 min, the absorbance decrease was linear during the whole observation time of 30 min indicating that MRP3 reconstituted in a lipid bilayer is stable under the conditions of the experiment. The basal ATPase activity of MRP3 in asolectin proteoliposomes determined from more than 20 independent experiments was (81 ± 12) nmol mg^{-1} min^{-1} and shows perfect agreement with results obtained by quantification of released inorganic phosphate [10,11].

3.2. Stimulation of ATPase activity by bile acids

Although bile acids are discussed as substrates for MRP3 [18], their effect on ATPase activity has not yet been studied extensively. Therefore, ATP hydrolysis by MRP3 was determined in presence of deoxycholate, cholate, glycocholate and taurocholate (Fig. 1B). Except for deoxycholate, ATPase activity increased first from a basal level of 80 nmol mg^{-1} min^{-1} to a plateau around 180 nmol mg^{-1} min^{-1} in a sigmoidal way, followed and partly overlaid by a steeper increase to more than 300 nmol mg^{-1} min^{-1}. For deoxycholate the plateau around 180 nmol mg^{-1} min^{-1} could not be observed. The steep increase at higher concentrations of bile acids is caused by permeabilization of the proteoliposomes allowing MRP3 with its nucleotide binding domain oriented towards the lumen of the proteoliposomes to increasingly contribute to the detected ATP hydrolysis. Therefore, we investigated the efflux of carboxyfluorescein from liposomes under the conditions of the assay and found the onset of membrane permeabilization at 0.1 mM for deoxycholate, 0.6 mM for both cholate and glycocholate, and 1.0 mM for taurocholate (vertical lines in Fig. 1B). These concentrations coincide with the end of the ATPase activity plateaus mentioned before. Hence, only data below these critical permeabilization concentrations result from intact proteoliposomes and can be used for estimating kinetic parameters. For taurocholate, cholate and glycocholate, ATPase activity indicated positive cooperativity of two interacting subunits and apparent K_M values in the submillimolar to low millimolar range (details of fitting in Fig. 1C/D; data summarized in Table 1). For deoxycholate no reliable kinetic parameters could be determined because of the early onset of membrane permeabilization.

3.3. Substrate translocation and stimulated ATPase activity for model substrates

For further investigations of both substrate translocation and stimulated ATPase activity, we focused on compounds that show no detrimental effect on membrane permeability and are known to be transported by MRP3. We chose $E_2$17βG as a glucuronide substrate, CDCF as a hydrophilic xenobiotic substrate, MTX and LTC4 as amphipathic substrates of xenobiotic and physiological origin, respectively.

In the presence of $E_2$17βG, ATPase activity increased from the common basal level to 191 nmol mg^{-1} min^{-1} nicely following the Hill equation ($R^2 = 0.998$) with positive cooperativity ($n = 2.0$) and an apparent K_M of 26 μM (Fig. 2A). Interestingly, we found the same cooperativity and concentration dependence ($n = 2.1$ and $K_M = 23$ μM with $R^2 = 0.975$) for the ATP-driven uptake of [^{3}H]$E_2$17βG into the lumen of proteoliposomes proceeding with a maximum transport rate of 20 nmol mg^{-1} min^{-1} (Fig. 2B).

For LTC4 we also observed a cooperative stimulation of ATP hydrolysis ($n = 2.1$ and $K_M = 1.1$ μM with $R^2 = 0.994$; Fig. 3) with the same concentration dependence than previous data on LTC4 uptake [10].

The correspondence of n and K_M between substrate translocation and stimulation of ATPase activity was also found for MTX (all data summarized in Table 1).

In contrast, CDCF did not stimulate ATP hydrolysis of MRP3 and showed a constant basal ATPase activity of 84 nmol mg^{-1} min^{-1} over four decades of CDCF concentration (Fig. 4A). However, the ATP-driven translocation of CDCF was concentration dependent and cooperative ($n = 2$) with an apparent K_M of 9.5 μM and a maximal transport rate of 28 pmol mg^{-1} min^{-1} (Fig. 4B).

4. Discussion

We assessed the effect of transport substrates on the ATPase activity of purified human MRP3 reconstituted into proteoliposomes for bile acids and sample compounds from the xenobiotic, glucuronide and leukotriene group of substrates. Except for CDCF, which had no

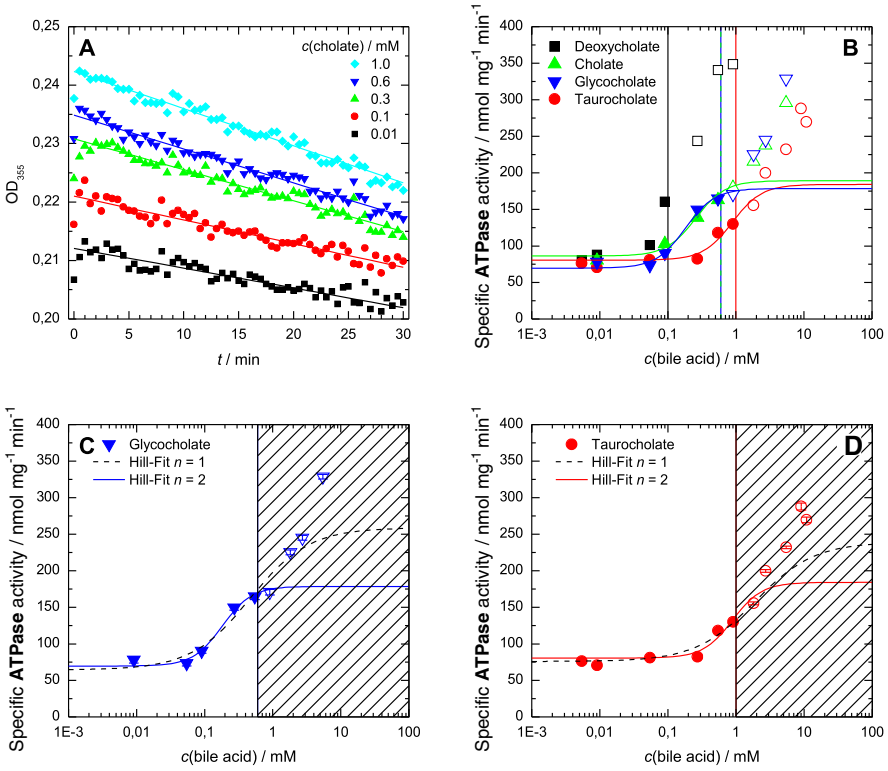


Fig. 1. Stimulation of ATPase activity of MRP3 by bile acids. MRP3 was reconstituted in asolectin proteoliposomes at a nominal lipid–protein ratio (w/w) of 50/1, and the rate of ATP hydrolysis after addition of bile acids was determined photometrically as NADH oxidation in a coupled enzyme assay. (A) Time-resolved absorbance at 355 nm for different concentrations of cholate (symbols) and linear regression (lines). Data from exemplary measurements is presented with arbitrary vertical offset for the sake of clarity. (B) Concentration-dependent stimulation of ATPase activity of MRP3 in proteoliposomes by bile acids. Specific ATPase activity was calculated from the slope of absorbance decrease at 355 nm as shown in (A). Vertical lines denote the critical permeabilization concentration for deoxycholate (0.1 mM), cholate/glycocholate (0.6 mM) and taurocholate (1.0 mM) as determined by carboxyfluorescein efflux. Data below these concentrations (solid symbols) was used for further analysis, data above (hollow symbols) disregarded. For taurocholate, cholate and glycocholate, fitting Eq. (1) (solid lines) to the data below the respective critical permeabilization concentration gave maximal ATPase activities in the range of 180–200 nmol mg^{-1} min^{-1}, a Hill coefficient of 2 and apparent K_M values in the sub-millimolar to low millimolar range as summarized in Table 1. (C) Curve fits for glycocholate with a Hill coefficient of 1 (dashed line, $R^2 = 0.870$, $\chi^2 = 16.6$) and 2 (solid line, $R^2 = 0.985$, $\chi^2 = 2.5$) are shown for comparison. The fit with a Hill coefficient of 2 describes the data better and indicates cooperativity. Data in the permeabilization region (shaded area) was not used for curve fitting. (D) The same for taurocholate comparing fits with a Hill coefficient of 1 (dashed line, $R^2 = 0.843$, $\chi^2 = 35.8$) and 2 (solid line, $R^2 = 0.920$, $\chi^2 = 9.7$) again favoring cooperativity. Data presented as mean of at least six independent experiments. Standard errors were small (≤5 nmol mg^{-1} min^{-1}) and are omitted for clarity in (B).

influence on the ATPase activity, all tested substrates stimulated ATP hydrolysis showing positive allosteric cooperativity of two interacting subunits (Hill coefficient of 2). The maximal rate of ATP hydrolysis was in the range of 180–200 nmol mg^{-1} min^{-1} and seems to be independent of the stimulating substance (Table 1). While the basal ($v_0 = (81 \pm 12)$ nmol mg^{-1} min^{-1}) and maximal ATPase activities are in good agreement with those reported for MRP3 in mixed lipid-detergent micelles [11], our results differ in terms of Hill coefficients, which inconsistently range from 0.5 to 2 in mixed micelles, and K_M values, which are up to one order of magnitude lower in mixed micelles. We attribute these differences to restricted access of substrates to the allosteric effector sites of MRP3 when located in an intact lipid membrane in contrast to mixed micelles. As cooperative interactions of the two NBDs are a conserved feature of ABC transporters [9], it was puzzling that previous studies on MRP3 in mixed micelles failed to observe these effects in a consistent way. Our data from proteoliposomes reveal near perfect cooperativity of two subunits for stimulated ATPase, though. Interestingly, the apparent K_M values for ATPase stimulation of MRP3 in proteoliposomes agree well with those for substrate translocation measured in plasma membrane vesicles (reviewed in [7]) and proteoliposomes (this study). These findings stress that it is always crucial to consider the reconstitution state of MRP3. Furthermore, as the reconstitution state has significant impact on the cooperativity of stimulated ATPase activity, one can speculate whether the underlying interactions are at least partly mediated by lipid–protein interactions, which are often overlooked in ABC transporter activity determination. Future experiments will therefore assess the cooperativity of stimulated ATP hydrolysis with respect to the lipid composition used for reconstitution. Asolectin was chosen in this study as it yielded basal ATPase activities comparable to the native environment of MRP3 (liver lipids) but with a more than twofold better reconstitution efficiency and reproducibility.

The case of CDCF showing no stimulation of ATP hydrolysis deserves special attention. CDCF as a small, very hydrophilic trianion is rather different from the usual MRP3 substrates comprising mostly lipophilic to amphipathic monoanions with a high logP value or their glucuronides. While these compounds are supposed to partition into the lipid membrane before binding to the TMD of MRP3 from within the lipid bilayer, CDCF is required to access the TMD directly from the aqueous phase. As the crystal structure of murine Pgp shows a portal for substrate entry open to both cytoplasm and the inner leaflet of the lipid bilayer [4], the substrate binding site of MRP3 might also be accessible from both aqueous media and membrane leaflet. The differing mode of access and its strong hydrophilicity might account for CDCF showing no stimulation of ATPase activity.

In addition, we assayed the translocation of $E_2$17βG, MTX, LTC4 and CDCF into the lumen of proteoliposomes by MRP3, finding an ATP-dependent uptake showing positive cooperativity of two subunits and maximal transport rates ranging from 28 pmol mg^{-1} min^{-1} for the slowly translocated CDCF to more than 2 μmol mg^{-1} min^{-1} for

Table 1
Kinetic parameters for stimulated ATPase and substrate transport of MRP3 obtained by Hill analysis and reported as mean ± S.E.

Substrate	ATPase			Transport		
	v_{max}/nmol mg^{-1} min^{-1}	K_M/μM	n	v_{max}/nmol mg^{-1} min^{-1}	K_M/μM	n
Cholate[a]	189 ± 21	257 ± 62	2[b]	–	–	–
Glycocholate[a]	179 ± 20	181 ± 51	2[b]	–	–	–
Taurocholate[a]	184 ± 31	888 ± 104	2[b]	–	–	–
Deoxycholate[a]	> 160	–	–	–	–	–
$E_2$17βG	191 ± 3	25.8 ± 2.2	2.0 ± 0.2	20.1 ± 1.8	23.2 ± 5.2	2.1 ± 0.3
LTC4	152 ± 7	1.1 ± 0.1	2.1 ± 0.4	4.7 ± 0.8[c]	1.7 ± 0.3[c]	2.3 ± 0.5[c]
MTX[d]	≥ 200	> 1000	2[b]	> 2000[c]	> 1000[c]	2.3 ± 1.7[c]
CDCF	84 ± 3[e]	–[e]	–[e]	0.028 ± 0.001	9.5 ± 1.1	2[b]

[a] Detergent properties of bile acids lead to low quality of ATPase data and prevented transport measurements due to permeabilization of liposomes.
[b] n was constrained to 0.5, 1, 2, 3 and 4.
[c] Data from previous study [10].
[d] Low data quality due to limited solubility (≤2 mM) of MTX.
[e] ATPase activity was independent of CDCF concentration; average rate reported instead of v_{max}.

the fast transported MTX (Table 1). As the transport rates presented here are based on the actual concentration of purified MRP3 in proteoliposomes, they appear up to 250 times higher than those reported for plasma membrane vesicles based on the total protein concentration in the membrane. However, the apparent K_M values for all tested substrates are in good agreement with data from plasma membrane vesicles [7,8,19]. Interference from other membrane proteins and transporters inherently present in cellular preparations are most likely the reason why MRP3-mediated substrate transport is often reported to follow non-cooperative Michaelis–Menten kinetic (f.e. [20,21]), while our data clearly reveal a cooperative Hill-type kinetic. Furthermore, it should be noted that – with the exception of CDCF – all tested substrates showed the same Hill coefficients and apparent K_M values for translocation and stimulation of ATPase activity.

The hypothetical transport cycle for MRP family transporters as reviewed in [9] features tight coupling between substrate translocation and ATPase activity. It starts with the binding of the substrate to the TMDs as the key step for activating the cooperating NBDs which subsequently dimerize by ATP binding thus causing conformational changes in the TMDs that eventually lead to substrate translocation. The initial state is then restored by ATP hydrolysis. Our observation of identical K_M values for substrate translocation and ATPase stimulation nicely supports this mechanism. It does, however, neither account for the high basal ATPase activity of MRP3 that comprises more than 40% of the maximal ATPase stimulation even in the absence of transport substrate, nor for the missing correlation between maximal ATPase stimulation,

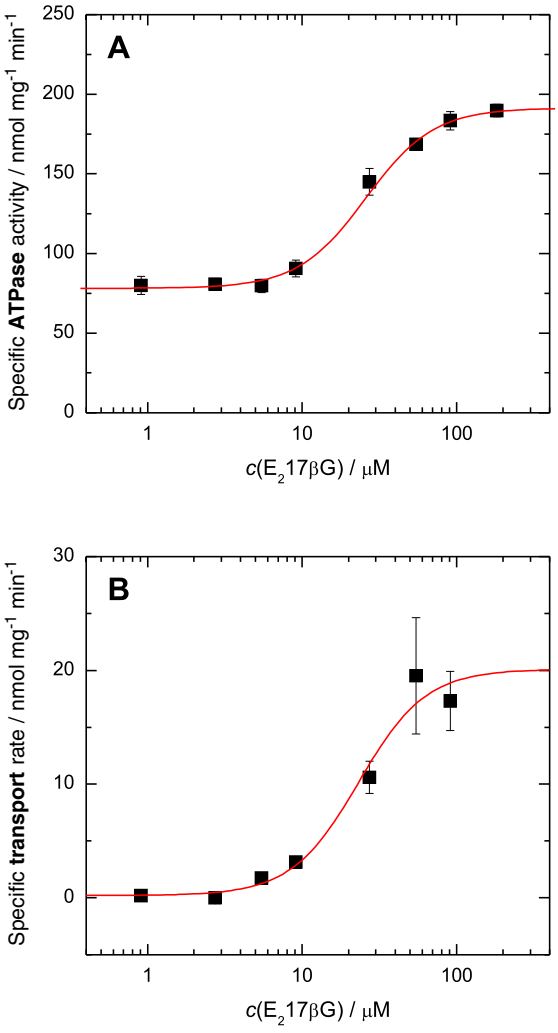


Fig. 2. Stimulated ATPase activity and substrate translocation by MRP3 in presence of $E_2$17βG. (A) Concentration-dependent stimulation of ATPase activity of MRP3 in proteoliposomes by $E_2$17βG. Fitting Eq. (1) (solid line) to the data gave a maximal ATPase stimulation of 191 nmol mg^{-1} min^{-1}, a Hill coefficient of 2.0 and an apparent K_M of 26 μM. Data presented as mean ± S.E. of at least six independent experiments. (B) ATP-driven uptake of [^{3}H]$E_2$17βG into the lumen of proteoliposomes containing MRP3. A fit of the Hill equation (solid line) gave a maximal transport rate of 20 nmol mg^{-1} min^{-1}, a Hill coefficient of 2.1 and an apparent K_M of 23 μM. Data presented as mean ± S.E. of at least three independent experiments.

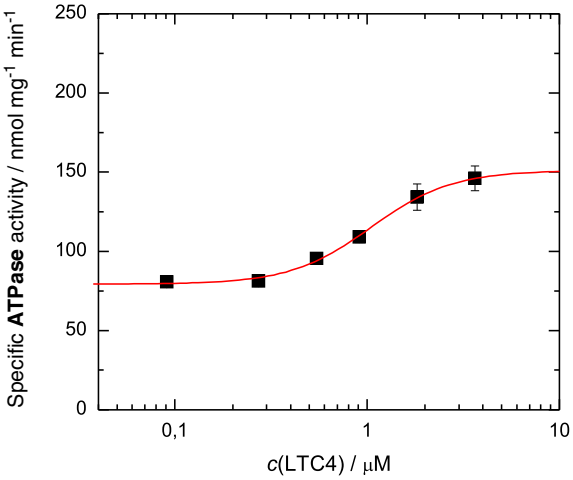


Fig. 3. Concentration-dependent stimulation of ATPase activity of MRP3 in proteoliposomes by LTC4. Fitting Eq. (1) (solid line) to the data gave a maximal ATPase stimulation of 152 nmol mg^{-1} min^{-1}, a Hill coefficient of 2.1 and an apparent K_M of 1.1 μM. Data presented as mean ± S.E. of at least six independent experiments.

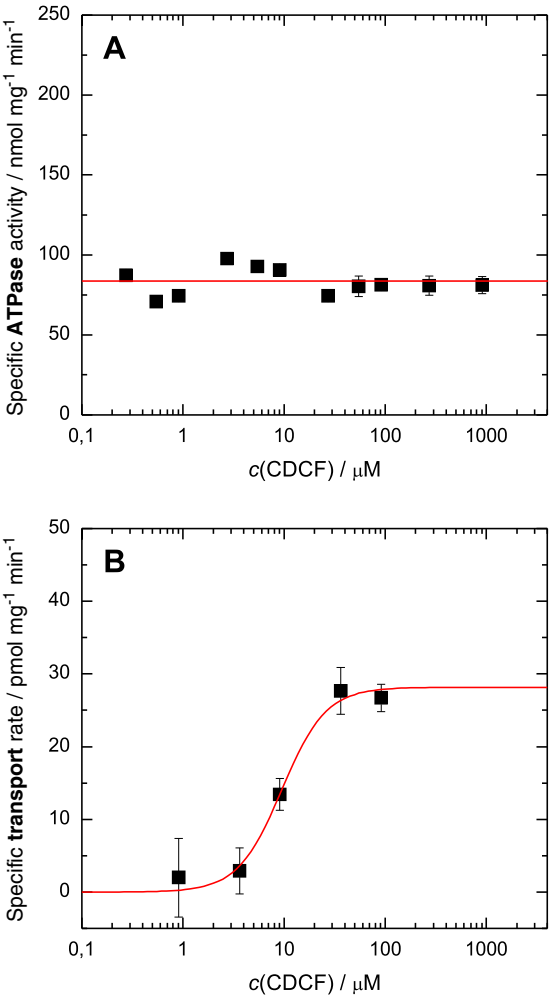


Fig. 4. ATPase activity and substrate translocation by MRP3 in presence of CDCF. (A) ATPase activity of MRP3 in proteoliposomes in presence of CDCF showing no concentration-dependent stimulation. The average ATPase activity (solid line) of 84 nmol mg^{-1} min^{-1} corresponds to the basal ATPase activity in the absence of substrate. Data presented as mean ± S.E. of at least six independent experiments. (B) ATP-driven uptake of CDCF into the lumen of proteoliposomes containing MRP3. A fit of the Hill equation (solid line) gave a maximal transport rate of 28 pmol mg^{-1} min^{-1}, a Hill coefficient of 2 and an apparent K_M of 9.5 µM. Data presented as mean ± S.E. of at least three independent experiments.

which is 180–200 nmol mg^{-1} min^{-1} regardless of the substrate, and the maximal transport rate which depending on the substrate varies over five orders of magnitude from tens of pmol mg^{-1} min^{-1} to µmol mg^{-1} min^{-1}. In case of Pgp, a similar effect is observed and explained by the experimentally verified partial uncoupling of ATPase and transport activity [6], where ATP hydrolysis and transport are uncoupled in absence of substrate and fully coupled at maximal activation. This uncoupling is additionally supposed to be intrinsic for the low substrate selectivity of Pgp [22]. MRP3 also shows low substrate selectivity, high basal ATPase activity and no correlation between maximal rate of substrate translocation and maximal ATPase stimulation. Hence, we believe that uncoupling can also be assumed for MRP3. As CDCF was shown to be transported in a concentration and ATP dependent manner but without stimulation of ATP hydrolysis, ATPase and transport cannot be fully uncoupled in MRP3, though. The basal ATPase activity is sufficient for substrate translocation indicating that conformational changes of the TMDs and ATPase activity of the NBDs are at least somewhat linked even in the absence of substrate. Using Krupkas terminology [22], we propose transport and ATPase activity of MRP3 to be half-coupled like in Pgp. One might speculate that CDCF due to its strong hydrophilicity cannot bind sufficiently to the TMDs to induce ATPase stimulation but enough to shuttle via the proposed basal TMD activity.

5. Conclusions

In conclusion, we showed that the reconstitution of purified MRP3 into proteoliposomes provides high-quality data for both substrate translocation and stimulated ATPase activity overcoming the drawbacks of plasma membrane vesicles from cellular preparations or purified protein in mixed lipid-detergent micelles. Our data clearly reveal cooperative interaction of two subunits during substrate translocation and stimulated ATP hydrolysis, both of which show identical apparent K_M values for all tested amphipathic substrates with the exception of the hydrophilic CDCF. These findings suggest that transport and ATPase activity of MRP3 are half-coupled.

Acknowledgments

We thank Ina L. Urbatsch (Texas Tech University) for generously providing the MRP3-expressing *P. pastoris* strain. Funding for this work was provided by SFB 858 of the Deutsche Forschungsgesellschaft (DFG) and the International Graduate School of Chemistry, Muenster. PS wishes to thank the Fond der chemischen Industrie (FCI) and the Studienstiftung des deutschen Volkes (SdV) for funding and support.

References

[1] M. Dean, A. Rzhetsky, R. Allikmets, The human ATP-binding cassette (ABC) transporter superfamily, Genome Research 11 (2001) 1156–1166.
[2] P. Borst, R.O. Elferink, Mammalian ABC transporters in health and disease, Annual Review of Biochemistry 71 (2002) 537–592.
[3] P. Borst, R. Evers, M. Kool, J. Wijnholds, The multidrug resistance protein family, Biochimica et Biophysica Acta 1461 (1999) 347–357.
[4] S.G. Aller, J. Yu, A. Ward, Y. Weng, S. Chittaboina, R. Zhuo, P.M. Harrell, Y.T. Trinh, Q. Zhang, I.L. Urbatsch, G. Chang, Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding, Science 323 (2009) 1718–1722.
[5] A.E. Senior, M.K. al-Shawi, I.L. Urbatsch, The catalytic cycle of P-glycoprotein, FEBS Letters 377 (1995) 285–289.
[6] M.K. Al-Shawi, M.K. Polar, H. Omote, R.A. Figler, Transition state analysis of the coupling of drug transport to ATP hydrolysis by P-glycoprotein, Journal of Biological Chemistry 278 (2003) 52629–52640.
[7] P. Borst, C. de Wolf, K. van de Wetering, Multidrug resistance-associated proteins 3, 4, and 5, Pfluegers Archiv 453 (2007) 661–673.
[8] H. Zeng, G. Liu, P.A. Rea, G.D. Kruh, Transport of amphipathic anions by human multidrug resistance protein 3, Cancer Research 60 (2000) 4779–4784.
[9] R.G. Deeley, C. Westlake, S.P. Cole, Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins, Physiological Reviews 86 (2006) 849–899.
[10] B. Zehnpfennig, I.L. Urbatsch, H.-J. Galla, Functional reconstitution of human ABCC3 into proteoliposomes reveals a transport mechanism with positive cooperativity, Biochemistry 48 (2009) 4423–4430.
[11] M. Chloupková, A. Pickert, J.Y. Lee, S. Souza, Y.T. Trinh, S.M. Connelly, M.E. Dumont, M. Dean, I.L. Urbatsch, Expression of 25 human ABC transporters in the yeast *Pichia pastoris* and characterization of the purified ABCC3 ATPase activity, Biochemistry 46 (2007) 7992–8003.
[12] N. Lerner-Marmarosh, K. Gimi, I.L. Urbatsch, P. Gros, A.E. Senior, 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, Journal of Biological Chemistry 274 (1999) 34711–34718.
[13] A. Hoffman, I. Urbatsch, P. Vogel, Nucleotide binding to the human multidrug resistance protein 3, MRP3, The Protein Journal 29 (2010) 373–379.
[14] G.B. Warren, P.A. Toon, N.J. Birdsall, A.G. Lee, J.C. Metcalfe, Reconstitution of a calcium pump using defined membrane components, Proceedings of the National Academy of Sciences of the United States of America 71 (1974) 622–626.
[15] E.R. Geertsma, N.A. Nik Mahmood, G.K. Schuurman-Wolters, B. Poolman, Membrane reconstitution of ABC transporters and assays of translocator function, Nature Protocols 3 (2008) 256–266.
[16] J.N. Weinstein, S. Yoshikami, P. Henkart, R. Blumenthal, W.A. Hagins, Liposome-cell interaction: transfer and intracellular release of a trapped fluorescent marker, Science 195 (1977) 489–492.
[17] P.P. Van Veldhoven, G.P. Mannaerts, Inorganic and organic phosphate measurements in the nanomolar range, Analytical Biochemistry 161 (1987) 45–48.
[18] T. Hirohashi, H. Suzuki, H. Takikawa, Y. Sugiyama, ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3), Journal of Biological Chemistry 275 (2000) 2905–2910.

[19] S. Pratt, V. Chen, W.I. Perry Iii, J.J. Starling, A.H. Dantzig, Kinetic validation of the use of carboxydichlorofluorescein as a drug surrogate for MRP5-mediated transport, European Journal of Pharmaceutical Sciences 27 (2006) 524–532.
[20] A. Bodó, É. Bakos, F. Szeri, A. Váradi, B. Sarkadi, Differential modulation of the human liver conjugate transporters MRP2 and MRP3 by bile acids and organic anions, Journal of Biological Chemistry 278 (2003) 23529–23537.
[21] N. Zelcer, T. Saeki, I. Bot, A. Kuil, P. Borst, Transport of bile acids in multidrug-resistance-protein 3-overexpressing cells co-transfected with the ileal Na+-dependent bile-acid transporter, Biochemistry Journal 369 (2003) 23–30.
[22] R.M. Krupka, Uncoupled active transport mechanisms accounting for low selectivity in multidrug carriers: P-glycoprotein and SMR antiporters, Journal of Membrane Biology 172 (1999) 129–143.