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Review

Transport of organic anions by multidrug resistance-associated protein in the erythrocyte★✪

Błażej Rychlik, Łukasz Pułaski, Adam Sokal, Mirosław Soszyński and Grzegorz Bartosz✉

Department of Molecular Biophysics, University of Łódź, Łódź, Poland

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The active transport of oxidized glutathione and glutathione S-conjugates has been demonstrated for the first time in erythrocytes and this cell remained the main subject of research on the "glutathione S-conjugate pump" for years. Further studies identified the "glutathione S-conjugate pump" as multidrug resistance-associated protein (MRP). Even though cells overexpressing MRP and isolated MRP provide useful information on MRP structure and function, the erythrocyte remains an interesting model cell for studies of MRP1 in its natural environment, including the substrate specificity and ATPase activity of the protein.

Studies on the function of multidrug resistance-associated protein (MRP) in erythrocytes had been initiated long before the protein was discovered and concerned the transport of oxidized glutathione (GSSG) and glutathione S-conjugates (GS-X) across the erythrocyte membrane. The ubiquitous tripeptide glutathione (γ-glutamyl-cysteinyl-

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✉To whom correspondence should be addressed: Department of Molecular Biophysics, University of Łódź, S. Banacha 12/16, 90-237 Łódź, Poland; phone (48 42) 635 4476; fax (48 42) 635 4473; e-mail: gbartosz@biol.uni.lodz.pl or gbartosz@widzew.net

Abbreviations: ABC, ATP-binding cassette; ARA, anthracycline resistance associated; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cMOAT, canalicular multispecific organic anion transporter; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DNP-SG, 2,4-dinitrophenyl-*S*-glutathione; GSH, γ-glutamyl-cysteinyl-glycine; GSSG, oxidized glutathione; GS-X, glutathione S-conjugate; LTC_4, leukotriene C_4; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TEMPO-glutathione, 2,2,6,6-tetramethyl-1-piperidinyloxy-4-maleimide-*S*-glutathione.

glycine; GSH) plays several functions in aerobic cells including the erythrocyte: 1) it is the main intracellular redox buffer maintaining protein –SH groups in the reduced form, 2) it is a substrate for reduction of hydrogen peroxide and organic peroxides by glutathione peroxidase, and 3) it is a substrate for spontaneous or glutathione *S*-transferase-catalyzed conjugation of xenobiotics. The first two classes of reactions produce oxidized glutathione (GSSG) while glutathione S-conjugates are formed in the third one. Experimental evidence for export of both these substrates across the erythrocyte membrane has accumulated over many years. GSSG is recycled to GSH by glutathione reductase but under oxidative stress conditions, when the reductive capacity of the cell is exceeded, or after inhibition of glutathione reductase, excessive GSSG is transported out of the cell. Transport of GSSG out of erythrocytes oxidatively stressed with hydrogen peroxide (Srivastava & Beutler, 1969a) and treated with chromate to inhibit glutathione reductase (Srivastava & Beutler, 1969b) was reported for the first time in late 60's. The transport has the characteristics of active transport. As a matter of fact, it was the first evidence of active transport of organic compounds across the erythrocyte membrane, suggesting the presence of other active transport system(s) different from the classic cation pumps. Our experiments demonstrated subsequently that export of GSSG from erythrocytes can be also induced by other oxidants such as organic hydroperoxides and nitrite (Lapshina & Bartosz, 1995).

Export of a glutathione S-conjugate (2,4-dinitrophenyl-*S*-glutathione; DNP-SG) from erythrocytes was demonstrated in early 80's (Board, 1981). This transport was found to be independent of known cation pumps in the erythrocyte and of membrane potential which suggested that, like in the case of GSSG, it is a primary active transport.

The idea of primary active nature of the transport of GSSG and GS-X across the erythrocyte membrane stimulated attempts to identify the membrane ATPase activity stimulated by these substances. The erythrocyte is an ideal object for such studies due to the ease of isolation of pure cell membranes in large amounts and relatively few and low ATPase activities of the membranes. These studies resulted in reports on GSSG- and DNP-SG-stimulated ATPase activities of the erythrocyte membrane, apparently corresponding to the active transporters of these compounds (Kondo *et al.*, 1987; 1989; Sharma *et al.*, 1990) and coined the notions of "glutathione S-conjugate pump" and "GSSG pump" (Ishikawa, 1992).

KINETICS OF GSSG AND GLUTATHIONE S-CONJUGATE EXPORT FROM ERYTHROCYTES

Although the presence of GS-X/GSSG pumps has been demonstrated in various cell types, the erythrocyte seemed to be a perfect cell for functional characterization of these pumps and to identify and isolate the pumps by classical biochemical methods. Whole-cell studies demonstrated that export of both GSSG and DNP-SG is inhibited by fluoride and vanadate. DNP-SG transport out of human erythrocytes was found to be inhibited by orthovanadate (IC_{50} = 80 μM), *N*-ethylmaleimide (IC_{50} = 200 μM) and intracellular Ca^{2+} (IC_{50} = 700 nM), but not by erythrocyte anion exchange inhibitors such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and to be independent of membrane potential and medium osmolarity. The transport rate increased with increasing extracellular pH in the range of 6.0–8.0; the activation energy of the process was about 74 kJ mol^{-1} (Pulaski & Bartosz, 1995a).

Determination of DNP-SG efflux from whole cells is based on spectrophotometric determination of the release of this compound to

extracellular medium. This method is simple and reproducible but lacks sensitivity. We proposed therefore more sensitive methods based on fluorimetric measurements of a glutathione conjugate of bimane (Pulaski & Bartosz, 1995b) and electron spin resonance measurements of the release of the paramagnetic conjugate 2,2,6,6-tetramethyl-1-piperidinyloxy-4-maleimide-*S*-glutathione (TEMPO-glutathione) (Pulaski & Bartosz, 1996).

Inside-out vesicles can be easily prepared from erythrocyte membranes in high yield and provide a much better system than whole cells for precise characterization of the kinetics of GSSG and GS-X transport. Such studies performed in various laboratories gave slightly divergent results but demonstrated that the transport is ATP-dependent, inhibited by vanadate, fluorides and thiol reagents, and that there are two kinetic components of the transport of both GSSG and DNP-SG (Kondo *et al.*, 1982; LaBelle *et al.*, 1986a; 1986b; Awasthi *et al.*, 1989; Akerboom *et al.*, 1992; Heijn *et al.*, 1992; Bartosz *et al.*, 1993). We found the presence of two kinetic components of DNP-SG transport not only in human but also in rat, pig and bovine erythrocytes (Bartosz *et al.*, 1993). A summary of our results is given in Table 1. The mutual inhibition of high-affinity DNP-SG transport and GSSG transport indicated the presence in the erythrocyte membrane of one transporter capable of transport of both GSSG and GS-X. We also found that strong physiological oxidants such as peroxynitrite (Soszynski & Bartosz, 1997) and hypochlorite (Soszynski *et al.*, submitted) inactivate the transport of GS-X in erythrocytes.

Table 1. Kinetic characteristics of GSSG and DNP-SG transport across the human erythrocyte membrane.

From (Bartosz et al., 1993).

	High-affinity DNP-SG transport	Low-affinity DNP-SG transport	High-affinity GSSG transport	Low-affinity GSSG transport
K_m	2.7 ± 0.2 μM	0.90 ± 0.09 mM	23 ± 8 μM	5 mM
V_{max} [nmol/(mg protein × h)]	6.7 ± 0.3	94 ± 6	6.3 ± 1.0	66
K_i for inhibition by GSSG	88 ± 11 μM	no inhibition		
K_i for inhibition by DNP-SG			3.3 ± 0.8 μM	not determined
Activation energy of transport [kJ mol^{-1}]	51 ± 6	55 ± 9		
% activity with GTP as a substrate (activity with ATP = 100%)	111 ± 7	88 ± 2		
% activity with UTP as a substrate (activity with ATP = 100%)	70 ± 3	63 ± 6		
% activity with CTP as a substrate (activity with ATP = 100%)	48 ± 3	39 ± 1		
% activity in the presence of 100 μM *o*-vanadate	19 ± 1	41 ± 8	13 ± 3	
% activity in the presence of 1 mM *N*-ethylmaleimide	95 ± 8	25 ± 12	99 ± 9	
% activity in the presence of 1 mM DTNB	51 ± 11	21 ± 6		
% activity in the presence of 1 mM GSH	143 ± 12	121 ± 15		

ATTEMPTS TO IDENTIFY THE GLUTATHIONE S-CONJUGATE PUMP

GSSG-stimulated and DNP-SG-stimulated erythrocyte membrane ATPase activities have been characterized. The GSSG-ATPase activity, studied by only one group was found to show two components of different affinities towards GSSG (Kondo *et al.*, 1987; 1989). Interestingly, we measured the GSSG-stimulated ATPase activity of human erythrocyte membranes but were unable to see two components on the Lineweaver-Burk plots while the kinetics of stimulation of erythrocyte membrane ATPase activity by DNP-SG was biphasic (Fig. 1). We found that the affinity of

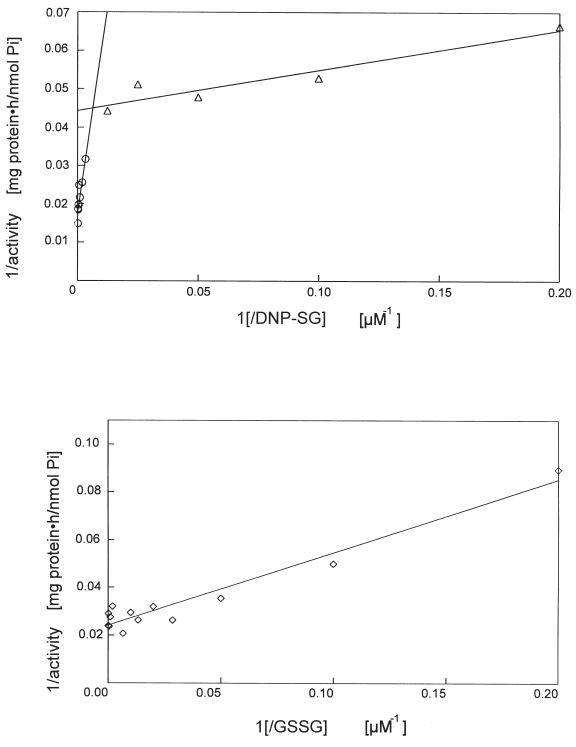

Figure 1. Lineweaver-Burk plots of the stimulation of erythrocyte membrane Mg^{2+}-ATPase activity by GSSG and DNP-SG (Frączak-Jaros *et al.*, submitted).

the erythrocyte membrane GS-X-ATPase to glutathione S-alkyl conjugates correlated with their hydrophobicity (Sokal *et al.*, 1995). Our observations suggested also that the interaction of the glutathione moiety with a protein is not a prerequisite for the stimulation of the erythrocyte membrane ATPase activity, as DNP-SG coupled to bovine serum albumin was as potent stimulator as DNP-SG alone (Sokal *et al.*, 1998).

The two kinetic components of GSSG-ATPase activities of low and high affinity for GSSG have been interpreted as representation of activities of different proteins which were subsequently isolated. A dimeric protein composed of 82 kDa and 62 kDa subunits was reported to be responsible for the high affinity GSSG-ATPase (Kondo *et al.*, 1987; 1989). Later on, the same group identified a protein of subunit molecular mass 28 kDa as corresponding to low-affinity GSSG-ATPase and DNP-SG-ATPase (Kondo *et al.*, 1993). Another group isolated from erythrocyte membranes (Sharma *et al.*, 1990; Awasthi *et al.*, 1998a; 1998b) and then from other sources (Sharma *et al.*, 1990; Awasthi *et al.*, 1991; Saxena *et al.*, 1992) a 38-kDa protein of DNP-SG-ATPase activity, immunochemically related to a 90-kDa GS-X transporter protein isolated from liver (Pikula *et al.*, 1994a; 1994b). The activity of the 38-kDa protein was not stimulated by GSSG (Sharma *et al.*, 1990).

All these studies failed to provide consistent and unequivocal proof of the molecular identity of the erythrocyte GS-X pump. However, identification of the protein was accomplished in other cell types.

MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN AS THE GLUTATHIONE S-CONJUGATE PUMP

In 1992 a novel protein contributing to multidrug resistance has been cloned which was overexpressed in H69AR cells selected from the parental cell line H69 by resistance to doxorubicin (Cole *et al.*, 1992; Cole & Deeley, 1993). This protein was distinct from P-glycoprotein (MDR1 protein) known to be the main cause of resistance of various cell lines towards some chemotherapeutics when

overexpressed, detected also in tumor samples taken from patients with a weak response to anticancer drugs (i.e. of the multidrug resistant phenotype, see a review (Fardel *et al.*, 1996)). Both proteins belong to the superfamily of ABC (ATP-binding cassette) transporters, a ubiquitous group of proteins sharing common motifs in the nucleotide binding folds involved in energy-dependent transmembrane transport in both eukaryotes and prokaryotes, for a review see (Holland & Blight, 1999).

The new protein, called MRP (multidrug resistance-associated protein) turned out to be also a membrane glycoprotein with a molecular mass of 190 kDa (Krischnamachary & Center, 1993). Further studies revealed that MRP is an active transporter able to diminish the intracellular accumulation of daunorubicin, vincristine and etoposide and conferring the resistance to a number of chemically non-related drugs (anthracyclines, *Vinca* alcaloids, colchicine and rhodamine 123) (Zaman *et al.*, 1994). Membrane vesicles transport studies proved that MRP transports also leukotriene C_4 (LTC_4), DNP-SG and GSSG as well as sulfates and glucuronides (Leier *et al.*, 1994), thus being functionally identical with the GS-X pump and GSSG pump. Interestingly, MRP was found to transport compounds which are not conjugates of xenobiotics (e.g. vincristine) with glutathione or other organic anions but this transport was dependent on the presence of GSH. This opened the field to suggestions that the GS-X pump may also function as a co-transporter of GSH and xenobiotics (Loe *et al.*, 1996).

MRP function is the final stage of the process of cellular detoxification of xenobiotics. The classical model of detoxification comprises two phases: activation and conjugation. The process of detoxification usually leads from hydrophobic compounds (which easily penetrate the cellular membrane) to more hydrophilic ones. This is achieved by conjugation to a hydrophilic moiety such as glucuronate or glutathione. Accumulation of products of xenobiotic conjugation in the cytoplasm is prevented by the third step of detoxification, i.e. the export of xenobiotic conjugates outside the cytoplasm. This step is the most important one for the metabolism of xenobiotics as some compounds are not detoxified but activated during the first phases of the process (as, e.g., aflatoxin B which is oxidized to chemically active epoxide that reacts easily with DNA bases) (Ishikawa, 1992). MRP functions as a transporter of conjugates of xenobiotics; perhaps it can also function as a co-transporter of non-modified xenobiotics and glutathione (Zaman *et al.*, 1994; Loe *et al.*, 1996).

Further studies demonstrated that in addition to MRP, renamed MRP1, several similar proteins occur in human cells. A liver canalicular membrane transporter of broad substrate specificity named cMOAT (canalicular multispecific organic anion transporter) proved to be 49% identical to MRP at the amino-acid sequence level and was renamed MRP2. A deficiency of this transporter leads to the Dubin-Johnson syndrome, an inheritable dysfunction of excretion of organic anions, including bilirubin glucuronates, into bile (Paulusma *et al.*, 1997). Another 3 members of the MRP family were identified in a panel of human cancer cell lines (Kool *et al.*, 1997) and finally MRP6, initially known as a fragment of sequence called *ARA* (from: anthracycline resistance associated) (Longhurst *et al.*, 1996) appeared. At the moment, six different proteins are identified, numbered from 1 to 6. It should be mentioned that a new nomenclature of ABC transporters has been proposed in which MRPs are to be called ABCCs 1 to 6 (compare web site: www.gene.ucl.ac.uk/users/hester/abc.html). MRPs are quite ubiquitously distributed in human organs and tissues, although most of them have tissue-specific localization (Kool *et al.*, 1997). Deletions of some of them may lead to inheritable disorders, as the Dubin-Johnson syndrome mentioned above, or *pseudoxanthoma elasticum* caused by mutations in *MRP6*

(*ABCC6*) gene (Bergen *et al.*, 2000). The functions of the members of the family may probably overlap as *mrp1*-double knockout mice do not reveal major pathological symptoms except for impaired inflammatory response (probably due to a decreased transport of leukotriene C_4) (Wijnholds *et al.*, 1997) and hypersensitivity to many chemotherapeutics (e.g. etoposide) caused by the weakening of the blood-testis, blood-kidney and blood-cerebrospinal fluid barriers (Wijnholds *et al.*, 1998; 2000).

The presence of MRP1 in human erythrocyte membranes has been demonstrated using immunochemical techniques. A comparison of its kinetic properties and substrate specificity determined in other cellular systems with the properties of the erythrocyte GS-X pump lead to the conclusion that MRP1 is responsible for the high affinity component of the GS-X pump (Pulaski *et al.*, 1996). This conclusion has been confirmed by studies on *mrp1*-knockout mice which lacked the high-affinity component of the GS-X pump (Wijnholds *et al.*, 1997). The identity of the second (low affinity) kinetic component of the erythrocyte GS-X pump still remains obscure. It seems probable that it corresponds to another protein of the MRP family.

ERYTHROCYTE AS A MODEL CELLULAR SYSTEM TO STUDY MRP FUNCTION

The occurrence of MRP1 in the erythrocyte makes this cell useful in studies of the function of this protein. The erythrocyte is a simple one-compartment model cell, containing only plasma membrane, without internal compartments, easy to obtain in any amount and easy to handle when preparing the membrane fraction. The human erythrocyte does not express P-glycoprotein which presence complicates the interpretation of transport measurements in other cell types. Of course, the erythrocyte model has its disadvantages. One of them is the unclear nature of the low-affinity transport of GS-X but its nature can be expected to be understood soon. Erythrocytes do not allow protein overexpression or deletion but a solution to this problem is the production of knockout organisms.

We estimated the functional size of MRP1 in erythrocyte membranes by radiation inactivation analysis. Our results indicated that MRP1 functions in this membrane as a dimer. Moreover, we demonstrated that the transporter responsible for the low-affinity DNP-SG transport has a similar functional size as MRP1 (Soszynski *et al.*, 1998).

Erythrocytes provide a good system for studies of the transport of various substrates of MRP1 and testing if various compounds are substrates of MRP by using MRP1 inhibitors (Fig. 2). We compared the efflux of a set of fluorescein derivatives from erythrocytes finding that 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), a fluorescent dye used as an intracellular pH indicator (Draper *et al.*, 1997) is an especially good MRP1 substrate (Table 2). This result is a suggestion for cytofluorimetric assessment of MRP1 overexpression (Rychlik and Bartosz, submitted).

The ATPase activity of P-glycoprotein, stimulated by various substrates and inhibited by vanadate has been the subject of numerous studies, mainly in membranes of cells overexpressing this protein (Ambudkar, 1998). Similarly, the ATPase activity of MRP1 was first demonstrated in membrane vesicles of MRP1-overexpressing cells (GLC4/ADR). This activity was slightly, but detectably, stimulated by DNP-SG, and also by some (iso)flavonoids (Hooijberg *et al.*, 1997). A purified and reconstituted protein, obtained by the His-tag technique, was also shown to possess the ATPase activity, but, surprisingly, only slightly stimulated by LTC_4, doxorubicin, *Vinca* alcaloids and other known substrates, except for GSSG (Chang *et al.*, 1997). These results were further confirmed by studies on the protein purified by immunoaffinity (Mao *et al.*, 1999).

The erythrocyte membrane is an interesting system to study the ATPase activity of MRP1 in its natural environment, against a relatively low background of basal Mg^{2+}-ATPase activity. Our studies of this activity revealed that some potential substrates and/or inhibitors of MRP1 as benzbromarone and probenecid stimulate this activity (Frączak-Jaros *et al.*, submitted). However, kinetic analysis indicated that anionic protonophores such as dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) are not substrates for the ATPase activity of MRP1 in erythrocytes (Sokal & Bartosz, 1998). We found also that the functional size of the DNP-SG-stimulated ATPase activity of the erythrocyte membrane corresponds to that of MRP1 as assessed by transport measurements (Soszynski *et al.*, 1998).

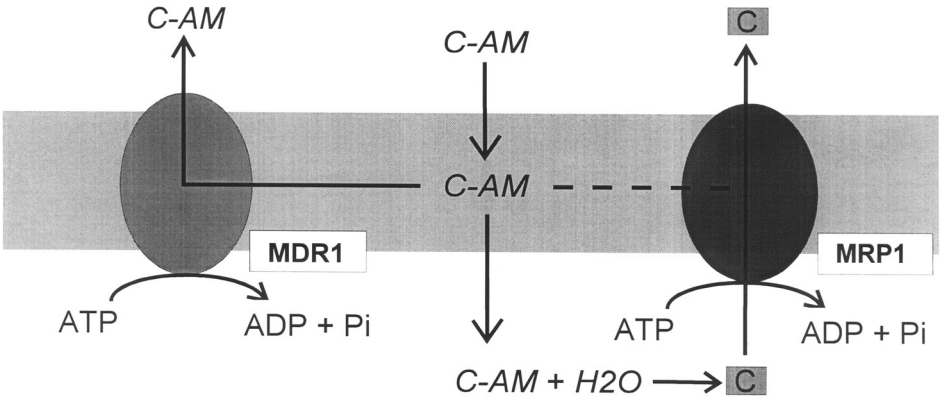

Figure 2. The principle of the measurement of organic anion transport from cells.

Non-fluorescent acetoxymethyl form of the dye (C-AM) easily penetrates the plasma membrane. When it reaches the cytoplasm it is cleaved by esterases to a free acid fluorescent form (C). If the MDR1 protein is present, it does not allow the ester to enter the cytoplasm by pumping it out already from the lipid bilayer, so the fluorescent product of hydrolysis is not formed (Homolya *et al.*, 1993). MRP1 pumps out the acid form of the dye (Hollo *et al.*, 1996). It is postulated that also the ester form of the dye is a substrate for MRP1 (dashed line). Additional evidence concerning the transporter involved comes from the studies of the effects of inhibitors of P-glycoprotein and MRP (Alvarez *et al.*, 1998).

In our opinion, the erythrocyte, the initial object of studies of the GSSG pump and GS-X pump, still remains an interesting object for studies of MRP in its natural membrane environment, including the transport specificity, ATPase activity and mechanism of action.

Table 2. The influence of MRP1 inhibitors on the efflux of fluorescent dyes from human erythrocytes

Dye/Inhibitor	Fluoride (up to 20 mM)	Vanadate (up to 200 μM)	Benzbromarone (up to 100 μM)	Probenecid (up to 1 mM)	Verapamil (up to 100 μM)
Fluorescein	–	–	–	–	–
Carboxyfluorescein	+	+	+	+	Stimulation
BCECF	++	++	++	++	+
Calcein	+	Not determined	+	++	+

– no effect, + weak inhibition, ++ strong inhibition

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