Organic anions exhibit distinct inhibition patterns on the low- K_m and high- K_m transport of *S*-(2,4-dinitrophenyl)glutathione through the human erythrocyte membrane

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The low- K_m and high- K_m components of *S*-dinitrophenylglutathione (DNPSG) uptake by inside-out vesicles of human erythrocytes show different pH profiles and inhibition properties with organic anions. Both components are competitively inhibited by various polyvalent anions, including glutathione conjugates, conjugated steroid hormones and bile salts, and bilirubin ditaurate. A variety of monovalent anions, including glucuronidated and sulphated drugs and taurocholate, inhibit the high- K_m system only. Taurocholate is taken up by the erythrocyte vesicles in an ATP-dependent manner. The anionic dyes fluorescein, Indocyanine Green and bromosulphophthalein

inhibit the low- K_m system competitively and the high- K_m system non-competitively. The study shows that interactions between different types of biologically occurring conjugates can occur at the level of the transport step out of erythrocytes. The kinetic properties suggest overlapping substrate specificities for the two systems, in which the low- K_m component is physiologically more important for transport of glutathione conjugates and polyvalent organic anions, whereas the high- K_m component is of significance for transport of monovalent organic anions. Low- and high- K_m transport of DNPSG is also observed in plasma membrane vesicles from rat, pig and bovine erythrocytes.

INTRODUCTION

Human erythrocytes are capable of active, ATP-dependent extrusion of glutathione thioethers (Board, 1981; Kondo et al., 1982; Awasthi et al., 1983; Eckert and Eyer, 1986) which contributes to cellular defence against reactive electrophiles. Two components of *S*-(2,4-dinitrophenyl)glutathione (DNPSG) transport have been identified in the human erythrocyte, one of low K_m and low capacity, and another of high K_m and high capacity (Eckert and Eyer, 1986; Akerboom et al., 1992). In inside-out vesicles from human erythrocytes we have observed that the high- K_m component is more sensitive to the action of thiol reagents, and that the low- K_m transport of DNPSG is competitively inhibited by GSSG and vice versa. The high- K_m system is not inhibited by GSSG. The low- K_m transport component probably represents a common high-affinity transporter for DNPSG and GSSG (Akerboom et al., 1992; Heijn et al., 1992).

DNPSG transport in the erythrocyte seems to be a primary active transport (LaBelle et al., 1986) mediated by a transporter capable of ATP hydrolysis whose enzymic activity can be measured as DNPSG-stimulated ATPase (LaBelle et al., 1988). The substrate specificity of the DNPSG ATPase is quite broad. Apart from various glutathione derivatives (Sharma et al., 1990), other structurally dissimilar organic anions such as bilirubin ditaurate, lithocholic acid 3-*O*-sulphate, 17 β -oestradiol 3-(β -D-glucuronide) and β -oestradiol 17-(β -D-glucuronide) can be substrates for the enzyme (Singhal et al., 1991).

In recent studies it was shown that sulphated and glucuronidated (divalent) bile salts, but not the (monovalent) parent compounds, inhibit both DNPSG efflux from rat hepatocytes (Oude Elferink et al., 1991) and the low- K_m ATP-dependent transport of GSSG in rat erythrocyte plasma membrane vesicles (Heijn et al., 1992). This prompted us to further examine the substrate specificities of the two DNPSG transport systems in

inside-out vesicles from human erythrocytes. We used, as a first approach, the kinetics of inhibition of both components of DNPSG transport by organic anions of various structures.

MATERIAL AND METHODS

[3 H]Glutathione (1 Ci/mmol) and [3 H]taurocholic acid (2.1 Ci/mmol) were purchased from NEN (Dreieich, Germany). Radioactively labelled DNPSG was synthesized from [3 H]glutathione and 1-chloro-2,4-dinitrobenzene (Merck, Darmstadt, Germany) as described previously (Akerboom et al., 1992). Non-radioactive DNPSG was prepared according to Vince et al. (1971). Using the same procedure, the *S*-(2,4-dinitrophenyl)-*N*-acetylcysteine thioether was synthesized by replacing glutathione with *N*-acetylcysteine. *S*-(bromosulphophthalein)glutathione was synthesized as described by Whelan et al. (1970). Bromosulphophthalein (BSP) was purchased from Fluka (Buchs, Switzerland). 1-[2-(Phenylcarbamoyl)phenyl]seleno- β -D-glucuronide acid (eb-selen glucuronide) was a gift from Nattermann/Rhone-Poulenc (Cologne, Germany). Nitrocellulose filters (0.65 μ m pore size; type 11306-25-N) were from Sartorius (Göttingen, Germany). Ultima Gold liquid scintillation fluid was from Packard (Groningen, The Netherlands). Other reagents were from Sigma (Deisenhofen, Germany).

Preparation of vesicles from erythrocytes and uptake measurements were performed as described elsewhere (Akerboom et al., 1992). The fraction of inside-out vesicles in the preparations was estimated by the acetylcholinesterase accessibility assay (Steck and Kant, 1974). The uptake data were expressed per mg of protein corresponding to inside-out vesicles.

Transport studies were performed employing the ATP-regenerating system of creatine kinase and phosphocreatine, and uptake rates were corrected for the corresponding blank sample values without ATP (Akerboom et al., 1992). In studies of

Abbreviations used: BSP, bromosulphophthalein; DNPSG, *S*-(2,4-dinitrophenyl)glutathione; DNP-N-taurine, *N*-(2,4-dinitrophenyl)taurine.

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percentage inhibition of low- K_m and high- K_m components, the concentrations of DNPSG were 1 μ M and 2 mM respectively. Kinetic studies of inhibition were performed with a concentration range of 1–25 μ M DNPSG for the low- K_m component and of 0.25–2 mM DNPSG for the high- K_m component.

Results are means $\pm$ S.E.M. from at least three independent experiments.

RESULTS

Two components of DNPSG transport in erythrocytes

Two components of DNPSG uptake are found in human, rat, pig and bovine erythrocytes (Table 1). The K_m values for both components in human erythrocytes, averaged from a large number of experiments ($n = 36$; Table 1), are in accordance with

Table 1 Kinetic parameters of the two components of DNPSG uptake by inside-out vesicles from human, rat, pig and bovine erythrocytes

Values have been corrected for the content of inside-out vesicles, and are means $\pm$ S.E.M.

Species	Low- K_m uptake		High- K_m uptake		n
	K_m^*	$V_m^\dagger$	K_m^*	$V_m^\dagger$	
Human	2.7 ± 0.2	6.7 ± 0.3	897 ± 86	94 ± 6	36
Rat	7.6 ± 2.6	20.5 ± 3.0	193 ± 37	48 ± 10	3
Pig	2.6 ± 0.3	5.9 ± 0.2	168 ± 14	20 ± 12	3
Bovine	1.5	2.4	480	111	2

* μ M.

† nmol/h per mg of protein corresponding to inside-out vesicles.

Table 2 Activation energies at 21–37 °C of DNPSG uptake and taurocholate uptake by inside-out vesicles from human erythrocytes

Transport	Activation energy (kJ/mol)
Low- K_m DNPSG	51 ± 6
High- K_m DNPSG	61 ± 5
Taurocholate	55 ± 9

Figure 1 pH-dependence of low- K_m (○) and high- K_m (●) DNPSG uptake by inside-out vesicles from human erythrocytes

The results are mean values from three experiments.

those reported by us previously (Akerboom et al., 1992). The low- K_m uptake is highest in the rat, whereas the high- K_m system is most active in human and bovine erythrocyte membranes. It is calculated that the low- K_m system predominates in bovine erythrocytes at DNPSG concentrations up to about 10 μ M, in humans and pig erythrocytes up to 70 μ M DNPSG, and in rat erythrocytes up to 260 μ M.

Activation energies for the two transport components are similar in vesicles from human erythrocytes (Table 2). The low- K_m process shows a pH optimum around 7.0, while the high- K_m transport maximum lies at pH 8.0 (Figure 1).

Inhibition of the low- K_m system

The low- K_m DNPSG transport system is competitively inhibited by several other glutathione conjugates bearing aromatic or aliphatic substituents (Table 3). The K_i values are below the K_m value for DNPSG, indicating that the transporter exhibits a high affinity for other glutathione conjugates. *S*-Methylglutathione is not inhibitory at concentrations up to 100 μ M, similar to the situation in heart and liver, in which it has been shown that glutathione conjugates with short side chains are poor inhibitors of ATP-dependent DNPSG transport (Ishikawa, 1989; Kobayashi et al., 1990). Likewise, inhibition of the low- K_m DNPSG transport system does not occur with the *S*-dinitrophenyl-*N*-acetylcysteine thioether.

A variety of organic anions bearing two or more negative charges act as competitive inhibitors of the low- K_m DNPSG uptake (Table 3). Bilirubin ditaurate and 3-*O*-sulphated bile salts are inhibitory with K_i values similar to those of the glutathione conjugates. These compounds have previously been suggested to share a common transport system with glutathione conjugates in liver (Oude Elferink et al., 1991; Awasthi et al., 1991) and erythrocytes (Singhal et al., 1991; Heijn et al., 1992). Furthermore, several polyvalent anionic dyes, including BSP, Indocyanine Green, fluorescein and carboxyfluorescein, competitively inhibit the low- K_m DNPSG transport system (Table 3). Rose Bengal is a non-competitive inhibitor.

Monovalent anions, even those that are structurally related to the polyvalent anions, are less effective or do not inhibit. The behaviour of oestradiol 3-glucuronide and oestradiol 17-glucuronide is remarkable in this respect. The 3-glucuronide does not inhibit low- K_m DNPSG uptake at concentrations up to 0.5 mM, whereas the 17-glucuronide isomer, bearing a free hydroxy group at the 3-position, exhibits competitive inhibition ($K_i = 170 \mu$ M). In addition, testosterone 17-glucuronide, which has an oxo-group at the 3-position, acts as a competitive inhibitor of low- K_m DNPSG uptake ($K_i = 130 \mu$ M).

The glucuronides and sulphates derived from 1-naphthol and *p*-nitrophenol, and also ebselen glucuronide and the uncharged glycoside ouabain, do not inhibit low- K_m DNPSG transport.

Inhibition of the high- K_m system

The glutathione conjugates and other organic anions shown to inhibit the low- K_m DNPSG transport system are also inhibitory to the high- K_m system (Table 3). The properties of the high- K_m DNPSG transport system, however, are different from those of the low- K_m system. Firstly, inhibition of the high- K_m system occurs with much higher K_i values. Secondly, a variety of monovalent organic anions which do not inhibit the low- K_m system competitively inhibit the high- K_m system. Thirdly, a number of dyes, including BSP, *S*-(BSP)glutathione, Indocyanine Green and fluorescein, which competitively inhibit the low- K_m system, inhibit the high- K_m system non-competitively. The latter compounds may not only act as substrate analogues, but enter a

Table 3 Effect of various organic anions on low- K_m and high- K_m DNPSG uptakeCharges are given at pH 7.4. C indicates competitive inhibition and N indicates non-competitive inhibition; $n = 3-6$ (except $n = 1$).

Compound	No. of charges		Concn. tested	Type of inhibition and K_i	
	Nega- tive	Posi- tive		Low- K_m uptake	High- K_m uptake
Glutathione derivatives					
S-Nitrobenzyl glutathione	2—	1 +	10 μ M 1 mM	C $2.1 \pm 0.5 \mu$ M	C 3.6 ± 1.1 mM
S-Hexylglutathione	2—	1 +	10 μ M 1 mM	C $1.3 \pm 0.6 \mu$ M	C 1.4 ± 0.3 mM
S-BSP-glutathione	6—	1 +	6 μ M 50 μ M	C $1.2 \pm 0.2 \mu$ M	N $60 \pm 15 \mu$ M
Leukotriene C4	3—	1 +	1.5 μ M 33 μ M	C $0.5 \pm 0.1 \mu$ M	C 30μ M*
S-Methylglutathione	2—	1 +	100 μ M	None	None
Dnp- N-acetylcysteine	1—		1 mM	None	C 1.2 ± 0.5 mM
Haem metabolites					
Bilirubin ditaurate	2—		5 μ M 10 μ M	C $2.4 \pm 0.3 \mu$ M	C $12 \pm 5 \mu$ M
Bile acids					
Taurocholic acid	1—		300 μ M	None	C $219 \pm 14 \mu$ M
Lithocholic acid 3-sulphate	2—		50 μ M	C $8.2 \pm 3.0 \mu$ M	C $123 \pm 23 \mu$ M
Glycolithocholic acid 3-sulphate	2—		50 μ M	C $5.6 \pm 0.8 \mu$ M	C $22 \pm 6 \mu$ M
Taurolithocholic acid 3-sulphate	2—		50 μ M	C $7.1 \pm 1.8 \mu$ M	C $27 \pm 7 \mu$ M
Steroid hormone derivatives					
Oestradiol 3-glucuronide	1—		500 μ M	None	C $779 \pm 526 \mu$ M
Oestradiol 17-glucuronide	1—		500 μ M	C $166 \pm 44 \mu$ M	C $344 \pm 122 \mu$ M
Testosterone 17-glucuronide	1—		300 μ M	C $126 \pm 24 \mu$ M	C $165 \pm 27 \mu$ M
Aromatic glucuronides					
Ebselen glucuronide	1—		500 μ M	None	C $180 \pm 65 \mu$ M
1-Naphthyl glucuronide	1—		1 mM	None	C $297 \pm 93 \mu$ M
p-Nitrophenyl glucuronide	1—		1 mM	None	C $876 \pm 44 \mu$ M
Aromatic sulphates					
1-Naphthyl sulphate	1—		200 μ M	None	C $616 \pm 225 \mu$ M
p-Nitrophenyl sulphate	1—		200 μ M 1.3 mM	None	C 1.6 ± 1.1 mM
Anionic dyes					
BSP	4—		30 μ M 50 μ M	C $16 \pm 6 \mu$ M	N $37 \pm 8 \mu$ M
Rose Bengal	2—		25 μ M	N $17 \pm 3 \mu$ M	N $9 \pm 2 \mu$ M
Indocyanine Green	2—	1 +	30 μ M	C $9.9 \pm 4.8 \mu$ M	N $12 \pm 4 \mu$ M
Fluorescein	2—		1 mM	C 1.7 ± 0.2 mM	N $885 \pm 277 \mu$ M
Carboxyfluorescein	3—		1 mM	C $489 \pm 23 \mu$ M	C $305 \pm 89 \mu$ M
Other organic anions					
DNP-N-taurine	1—		200 μ M 1 mM	None	C 1.1 ± 0.9 mM
Ouabain	0		100 μ M	None	None

chemical reaction with the transporter. For instance, BSP, having reactive bromine residues, may covalently bind to thiol groups. It is known that the high- K_m transport activity especially is susceptible to thiol reagents (Akerboom et al., 1992), explaining the non-competitive type of inhibition of this component by BSP. The multiple halogenated compound Rose Bengal inhibits both transporters non-competitively, possibly on the same basis.

Typical substrates for the monocarboxylate transporter of the erythrocyte membrane, including lactate (2 mM) and benzoate (2 mM) (Deuticke, 1989), do not interact with DNPSG transport (results not shown), suggesting that a certain hydrophobicity is also required for interaction with the high- K_m transporter. This could explain why GSSG (Akerboom et al., 1992) and S-methylglutathione (Table 3) are not inhibitory, and why the

conjugates of 1-naphthol exhibit lower K_i values than those of p-nitrophenol (Table 3).

DISCUSSION

The data presented suggest that efficient interaction with the low- K_m transporter is favoured by the presence of at least two negative charges on the molecule. However, the negative charges cannot be the only determinant for the interaction, as demonstrated by the inability of GSH and S-methylglutathione to inhibit low- K_m DNPSG uptake (Table 3; Akerboom et al., 1992). As discussed by Ishikawa (1989, 1990) some additional, perhaps hydrophobic, interaction must also be of importance. In recent studies Heijn et al. (1992) came to a similar conclusion. They

reported that inhibition of low- K_m GSSG transport is strongest by the divalent 3-*O*-sulphated and 3-*O*-glucuronidated derivatives of the monohydroxylated bile salt lithocholate, and that upon introduction of further hydroxy groups into the hydrophobic region the inhibitory potency decreases. Interestingly, the monovalent tauroolithocholate, which is non-esterified at the 3-hydroxy position, also showed some inhibition. Analogously, hydrophobic interactions may be involved in the inhibition of the low- K_m DNPSG transport by the monovalent oestradiol and testosterone 17-glucuronides (Table 3).

Competitive inhibition of DNPSG transport by the different compounds raises the question of whether they could act as substrates for the transporters. Livers from mutant rats in which the biliary excretion of the divalent 3-*O*-sulphated and glucuronidated bile acids, bilirubin diglucuronide, GSH, GSSG and glutathione conjugates was defective showed normal excretion of the monovalent bile acids, and separate multispecific transport pathways were proposed (Jansen et al., 1987; Kuipers et al., 1988, 1989; Oude Elferink et al., 1989; Nishida et al., 1992). For the erythrocyte, the physiological significance of transport of most of these compounds, including the bile salts, through the plasma membrane seems doubtful. However, upon testing the uptake of taurocholate by the erythrocyte membrane vesicles, we observed a significant ATP-dependent transport. The Lineweaver-Burk plot showed the presence of only one component with a K_m of $78 \pm 20 \mu\text{M}$ and a V_{max} of $2.8 \pm 0.6 \text{ nmol/h}$ per mg of protein ($n = 6$) (results not shown). The activation energy for taurocholate transport amounts to 55 kJ/mol, which is similar to that for ATP-dependent DNPSG transport (Table 2). In canalicular membrane vesicles from rat liver we have shown that taurocholate interferes with DNPSG transport and vice versa (Akerboom et al., 1991), and this was also reported for *p*-nitrophenyl glucuronide (Kobayashi et al., 1991). Both taurocholate and *p*-nitrophenyl glucuronide competitively inhibit the high- K_m DNPSG transporter in the erythrocyte, so that this transporter might be related to the liver enzyme and exhibit multispecific transport.

From studies on the low- K_m ATP-dependent transport of GSSG in erythrocyte membrane vesicles, Heijn et al. (1992) concluded that the transporter represents a multispecific ATP-dependent organic anion transport system, which is similar if not identical to the hepatocanalicular transporter. The immunological cross-reactivity of the erythrocyte GSSG ATPase (Kondo et al., 1989) and DNPSG ATPase (Awasthi et al., 1991) with the corresponding liver enzymes supports this theory. However, the situation seems to be more complex, as a number of differences are apparent. Firstly, normal transport rates for both GSSG and DNPSG are observed in intact erythrocytes and inside-out erythrocyte membrane vesicles from Dubin-Johnson patients and in mutant rats, whereas in these species the hepatocanalicular transport of both forms of glutathione is defective (Board et al., 1992). Secondly, the canalicular transport system is sensitive to thiol reagents and is competitively inhibited by taurocholate (Akerboom et al., 1991), whereas the low- K_m DNPSG transporter in erythrocytes is not (Akerboom et al., 1992; Table 3). The high- K_m DNPSG transporter in the erythrocyte is different from the hepatic canalicular transporter with respect to its lack of inhibition by GSSG (Table 3; Akerboom et al., 1991).

The present paper shows that the erythrocyte plasma membrane contains two components of ATP-dependent DNPSG transport with different inhibition kinetics towards organic anions. The two components may represent separate transport systems with overlapping substrate specificities. Our data indicate that the low- K_m system is involved in the transport of glutathione conjugates at physiological concentrations and possibly also in the transport of divalent organic anions. Monovalent organic anions, including bile salts and sulphated and glucuronidated drugs, have no effect on the low- K_m transport system. Transport of these compounds might proceed via the high- K_m transport component, which represents an organic anion translocator with broader specificity.

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