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Low- and high-K_m transport of dinitrophenyl glutathione in inside out vesicles from human erythrocytes

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Kinetic studies on the low- and high-K_m transport systems for *S*-2,4-dinitrophenyl glutathione (DNP-SG) present in erythrocyte membranes were performed using inside-out plasma membrane vesicles. The high-affinity system showed a K_m of 3.9 μM a V_{max} of 6.3 nmol/mg protein per h, and the low-affinity system a K_m of 1.6 mM and a V_{max} of 131 nmol/mg protein per h. Both uptake components were inhibited by fluoride, vanadate, *p*-chloromercuribenzoate (pCMB) and bis(4-nitrophenyl)dithio-3,3'-dicarboxylate (DTNB). The low-K_m uptake process was less sensitive to the inhibitory action of DTNB as compared to the high-K_m process. *N*-Ethylmaleimide (1 mM) inhibited the high-K_m process only. The high-affinity uptake of DNP-SG was competitively inhibited by GSSG (K_i = 88 μM). Vice versa, DNP-SG inhibited competitively the low-K_m component of GSSG uptake (K_i = 3.3 μM). The high-K_m DNP-SG uptake system was not inhibited by GSSG. The existence of a common high-affinity transporter for DNP-SG and GSSG in erythrocytes is suggested.

Introduction

Human erythrocytes are capable of extrusion of glutathione disulfide (GSSG) [1–5] and of glutathione thioethers, called conjugates [6–11]. Transport of these glutathione species is an essential part in cellular defense against oxidants and reactive electrophiles; it requires ATP. In inside-out vesicles of human erythrocyte membranes the existence of two components for GSSG transport has been demonstrated, one of high affinity and low capacity, and another of low affinity and high capacity [4,5]. In contrast, so far only one component of DNP-SG transport was identified in inside-out vesicles, with K_m of 0.3–0.9 mM [7,9]. Eckert and Eyer [11] demonstrated, however, that DNP-SG export from intact erythrocytes has also two components, one with a K_m of 1.4 μM and one with a K_m of 0.7 mM.

Controversy persists concerning the relationship between GSSG and DNP-SG transport in the erythrocyte. Kondo et al. [7] suggested that the high-K_m GSSG transport system extrudes also DNP-SG since the glutathione conjugate behaved as a competitive inhibitor. Also in liver and heart competition of transport has been shown for these glutathione species [12–15]. However, LaBelle et al. [9,10] found that DNP-SG transport is not inhibited by GSSG and proposed that DNP-SG is transported via a separate system.

GSSG-dependent ATPases of low and high affinities for GSSG have been isolated from erythrocyte membranes [16]. The low-K_m high-affinity GSSG-ATPase was shown to mediate ATP-dependent GSSG uptake when incorporated into liposomes [17]. A DNP-SG-dependent ATPase has also been isolated from erythrocyte membranes [18]. This ATPase was stimulated by anionic conjugates of bilirubin and bile acids, but not by GSSG [18,19].

The present study was undertaken to examine the properties of the low- and high-K_m transport systems for DNP-SG and their relationship to GSSG transport in human erythrocytes. The data obtained indicate the existence of a common high-affinity transport system for GSSG and glutathione conjugates. The high-K_m

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Abbreviations: pCMB, *p*-chloromercuribenzoate; DNP-SG, *S*-2,4-dinitrophenyl glutathione; DTNB, bis(4-nitrophenyl)dithio-3,3'-dicarboxyiate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

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system for DNP-SG does not transport GSSG, consistent with previous reports [9,10]. The low-K_m component of DNP-SG transport, previously not described in inside-out vesicles of erythrocyte membranes, may be of major physiological significance because thioethers at low concentrations bind to other glutathione binding sites inhibiting metabolic reactions, e.g. GSSG-reductase [14,20].

Materials and Methods

[^{3}H]Glutathione was obtained from NEN, (Dreieich, Germany). Nitrocellulose filters (0.45 μm; Type 11306-25-N) were from Sartorius (Göttingen, Germany). Ultima Gold™ liquid scintillation fluid was from Packard (Groningen, Netherlands). Silica gel TLC plates (Kieselgel 60) and 1-chloro-2,4-dinitrobenzene were from Merck (Darmstadt, Germany). GSH and GSSG were from Boehringer (Mannheim, Germany), QAE-Sephadex from Pharmacia (Freiburg, Germany) and other reagents from Sigma (Deisenhofen, Germany).

^{3}H-Labeled glutathione disulfide was prepared from [^{3}H]glutathione (specific activity of 1 Ci/mmol) by oxidation with a 3-fold excess of H_2O_2 and purified by preparative silica gel TLC [13]. [^{3}H]DNP-SG was synthesized enzymatically according to Awasthi et al. [21] and purified by QAE-Sephadex chromatography and preparative silica gel TLC.

Inside-out vesicles were prepared from human erythrocytes by a modification of the method of Steck and Kant [22]. The modification consisted of employment of 0.4 mM Tris-HCl (pH 8.0) containing 0.2 mM EDTA instead of phosphate and inclusion of 250 mM sucrose in the vesiculation medium which permitted a better compensation for the osmolarity of the media used for transport studies.

Acetylcholinesterase accessibility assay [22] demonstrated that the preparations contained 59% ± 10% inside-out vesicles. The uptake values measured were corrected for this value and expressed per mg protein corresponding to inside-out vesicles.

In studies of the thermal inactivation of uptake, the vesicles were preincubated in 250 mM sucrose/0.2 mM EDTA/0.4 mM Tris-HCl (pH 8.0), at temperatures indicated in the text, for 15 min prior to transport measurements. Transport activities were measured at 37°C, as specified below.

The incubation medium for transport studies contained 250 mM sucrose, 20 mM $MgCl_2$, 1 mM ATP, 30 mM creatine phosphate, 200 U/ml creatine kinase, 10 mM Tris-HCl (pH 7.4), GSSG or DNP-SG and additives as indicated. Uptake rates were corrected for the corresponding blank samples without ATP. For the comparison of nucleotide specificity of the uptake, the ATP regenerating system of creatine phosphate and creatine kinase was omitted and appropriate nucleotides were present at a concentration of 3 mM. The medium contained 1 μCi/ml of [^{3}H]DNP-SG or [^{3}H]GSSG made up to the required concentrations with the unlabelled compounds. The samples were preheated to and then incubated at 37°C. The uptake was started by addition of vesicles to give a final protein concentration of 0.8 mg/ml. Incubation times were 1 h for the low-K_m GSSG uptake and high-K_m DNP-SG uptake, and 15 min for the low-K_m DNP-SG uptake, so that uptake was proportional to incubation time. The reaction was stopped by dilution of 20-μl aliquots of the suspensions with an ice-cold stop solution (250 mM sucrose, 100 mM NaCl, 10 mM Tris-HCl (pH 7.4)) followed by immediate filtration through a 0.45-μm nitrocellulose filter prewashed with 3 ml of water and rapid washing with 2 ml of ice-cold stop solution. The radioactivity retained on the filter was counted in a Beckman LS 1801 liquid scintillation counter.

Results and Discussion

GSSG transport

Two components of the GSSG uptake by inside-out erythrocyte membrane vesicles were distiguished, one with low K_m and low capacity, and another with high K_m and high capacity (Fig. 1, top), in agreement with data of Kondo et al. [4,5]. Our best-fit K_m values were 23 μM and 5 mM, respectively (Table I). The K_m of the low-K_m component for ATP was 131 μM, a value significantly lower than that (0.63 mM) reported previously for GSSG transport [4,5] but similar to that (80 μM) found for the low-K_m GSSG-ATPase activity [16]. It may be noted that in our experiments we employed the ATP-regenerating system of creatine phosphate and creatine kinase to prevent ATP exhaustion especially at low ATP concentrations.

Measurements of the high-K_m component of the GSSG uptake were troublesome because of the relatively high ATP-independent uptake and binding, and this component was not further studied.

DNP-SG transport

Measurements of the concentration dependence of DNP-SG uptake over a broad concentration range demonstrate the presence of a low-K_m and a high-K_m component like for GSSG (Fig. 1, bottom). The respective apparent K_m values for DNP-SG were 3.9 μM (V_{max} 6.3 nmol/mg membrane protein per h) and 1.6 mM (V_{max} 131 nmol/mg membrane protein per h) (Table I). The existence of two transport systems has been previously suggested by Eckert and Eyer in studies of DNP-SG extrusion from intact erythrocytes preloaded with 1-chloro-2,4-dinitrobenzene [11]. The low-K_m process of DNP-SG transport has so far not been further characterized.

TABLE I

Apparent kinetic constants for DNP-SG and GSSG transport in human erythrocytes

* Unit: nmol/mg protein per h. n.d., not determined. For conditions of determination of K_m for DNP-SG and GSSG see Fig. 1. K_m values for ATP were determined at: [a] 3.5 μM DNP-SG, [b] 2 mM DNP-SG, [c] 10 μM GSSG and ATP ranging from 50 μM to 2 mM. K_i values were determined at 200 μM GSSG and 3 μM DNP-SG (cf Fig. 2). Data represent means ± S.E. (n = 3–7).

	DNP-SG uptake		GSSG uptake	
	low-K_m process	high-K_m process	low-K_m process	high-K_m process
K_m	3.9 ± 0.7 μM	1.6 ± 0.6 mM	23 ± 8 μM	5 mM
V_{max} *	6.3 ± 1.3	131 ± 53	6.3 ± 1.0	66
K_m for ATP	32 ± 3 μM [a]	83 ± 22 μM [b]	131 ± 15 μM [c]	n.d.
K_i for GSSG	88 ± 11 μM	no inhibition	–	–
K_i for DNP-SG	–	–	3.3 ± 0.8 μM	n.d.

The apparent K_m values found for ATP were 32 μM and 83 μM for the low-K_m and high-K_m components, respectively (Table I). Again, these values are much lower than those measured previously for the DNP-SG uptake of 0.76 mM [7] and 0.93 mM [9] in the absence of an ATP-regenerating system. Pyrimidine nucleotides (CTP and UTP) were less effective in stimulating both components of the DNP-SG uptake than purine nucleotides (ATP and GTP) (Table II).

Both components of the DNP-SG uptake were found to be inhibited by *o*-vanadate and fluoride (Table III). Inhibition of the extrusion of GSSG [1] and DNP-SG [8,11] from intact erythrocytes by fluoride has been

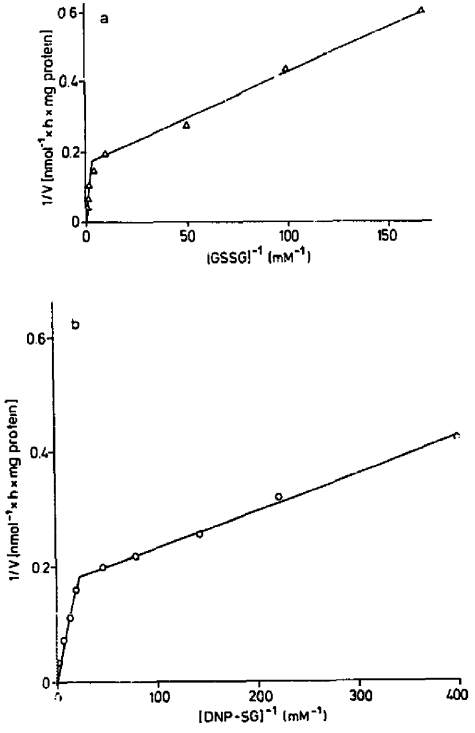

Fig. 1. Lineweaver-Burk plot of the GSSG (a) and DNP-SG (b) uptake by inside-out vesicles of human erythrocyte membranes. Transport measured at 4 μM–2 mM DNP-SG and 6 μM–12 mM GSSG, respectively. Data from a single representative experiment.

TABLE II

Nucleotide specificity of the two components of DNP-SG uptake

Low-K_m and high-K_m uptake were determined at 3.5 μM and 2 mM DNP-SG, respectively. Values are means ± S.E. from three experiments.

	% of uptake with ATP	
	low-K_m process	high-K_m process
GTP	111 ± 7	88 ± 2
UTP	70 ± 3	63 ± 6
CTP	48 ± 3	39 ± 1

TABLE III

Effects of inhibitors / activators on the low-K_m and high-K_m components of DNP-SG uptake and on the high-affinity component of GSSG uptake

The low-K_m and high-K_m DNP-SG uptake systems were studied at 3.5 μM and 2 mM DNP-SG, respectively. GSSG uptake was determined at 10 μM GSSG. Mean ± S.E. (n = 3–5).

	% of control		
	low-K_m DNP-SG uptake	high-K_m DNP-SG uptake	low-K_m GSSG uptake
NaF (25 mM)	1 ± 1	15 ± 8	2 ± 2
o-Vanadate (0.1 mM)	19 ± 1	41 ± 8	13 ± 3
NEM (1 mM)	95 ± 8	25 ± 12	99 ± 9
DTNB (1 mM)	51 ± 11	21 ± 6	
pCMB (0.1 mM)	4 ± 3	29 ± 11	
DTT (1 mM)	101 ± 3	73 ± 5	
GSH (1 mM)	143 ± 12	121 ± 15	

ascribed to energy depletion of cells. The present data obtained in studies of erythrocyte membrane vesicles demonstrate that the effect of fluoride is due, however, to the direct interaction of this anion with the transporters of these compounds, as observed for other magnesium-dependent enzymes [23].

The high-K_m DNP-SG uptake system was substantially inhibited by thiol reagents NEM, DTNB and pCMB. The low-K_m uptake system showed a different inhibition pattern, being less sensitive to DTNB, and not inhibited by NEM (1 mM). GSH induced a slight stimulation of DNP-SG uptake, which may be ascribed to a re-reduction of oxidized thiol groups of the transporters. The low-K_m component of GSSG uptake showed inhibition properties similar to the low-K_m DNP-SG uptake process. Unfortunately, it was not possible to examine the effects of DTNB, GSH and DTT on GSSG uptake as these compounds entered exchange reactions with GSSG, as detected by TLC analysis (not shown).

Mutual competitive inhibition of the high-affinity DNP-SG and GSSG uptake

We found inhibition of the low-K_m DNP-SG uptake system by GSSG and of the low-K_m GSSG uptake system by DNP-SG. In both cases the inhibition was competitive (Fig. 2). The apparent K_i of GSSG for the inhibition of DNP-SG uptake was 88 ± 11 μM and that of DNP-SG for the inhibition of GSSG uptake was 3.3 ± 0.8 μM (mean $\pm$ S.E., $n = 7$). We did not observe any inhibition of the high-K_m DNP-SG uptake system by GSSG at concentrations of up to 12 mM (data not shown), in agreement with the results of LaBelle et al. [9,10].

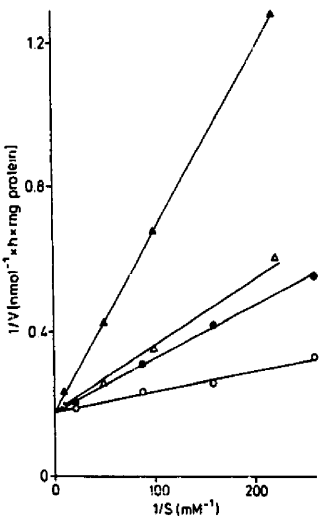

Fig. 2. Mutual competitive inhibition of the high-affinity uptake of DNP-SG and GSSG. Symbols are: (○), DNP-SG; (●), DNP-SG with 200 μM GSSG; (△), GSSG; (▲), GSSG with 3 μM DNP-SG. Mean values from three experiments.

TABLE IV

Thermal inactivation of DNP-SG and GSSG uptake

Vesicles preincubated at indicated temperatures; transport was measured at 37°C under the conditions specified in Table III. Mean values from two experiments.

Temp. (°C)	% of control		
	low-K_m DNP-SG uptake	high-K_m DNP-SG uptake	low-K_m GSSG uptake
42.0	27	26	33
43.0	16	16	23
44.0	8	13	12

Thermal inactivation of DNP-SG and GSSG uptake

All components of DNP-SG and GSSG uptake showed a considerable thermal sensitivity being inactivated by preincubation of vesicles at temperatures of 42–44°C (Table IV). The considerable thermal sensitivity may be a way of differentiation of the transport of GSSG and glutathione derivatives from other transport systems. Anion exchange mediated by the band 3 protein of the erythrocyte membrane does not show significant inactivation up to temperatures of about 60°C [24].

Relationship between DNP-SG and GSSG uptake

Several lines of evidence suggest that the low-K_m DNP-SG transport process and the low-K_m GSSG transport process in the human erythrocyte is mediated by the same transporter: (i) GSSG and DNP-SG exhibit mutual competitive inhibition, (ii) the low-K_m transport of GSSG and DNP-SG exhibits a similar sensitivity to different inhibitors, notably NEM, (iii) both glutathione species show identical values of maximal velocity of the uptake (Table I, Fig. 2), and (iv) the K_m for DNP-SG transport equals the K_i of DNP-SG for inhibition of low-K_m GSSG transport. It is generally assumed that if two compounds share the same transporter, the K_m value for the transport of one compound should be equal to the K_i value for this compound when acting as a competitive inhibitor of the transport of the other compound [25]. In the case of GSSG, the apparent value of K_i for the inhibition of low-K_m DNP-SG uptake (88 μM) was higher than the apparent K_m value for the low-K_m transport system of glutathione disulfide (23 μM) found by us (Table I) but similar to that reported by others, both for transport (100 μM) [4,5] and for GSSG-ATPase activity (130 μM) [16].

In contrast to the low-K_m component, the high-K_m component of DNP-SG transport is not inhibited by

GSSG. Thus, these results offer an explanation for the apparent contradictory interpretations in literature regarding the existence of a common transport system for DNP-SG and GSSG. In particular, they make clear the reported lack of inhibition of DNP-SG transport by GSSG [9,10] observed under conditions (160 μM DNP-SG) where the high-K_m process must have been responsible for most of the DNP-SG uptake.

In canalicular membrane vesicles from rat liver ATP-dependent DNP-SG transport is inhibited by GSSG [26]. We have recently shown that the inhibition is competitive, and that ATP-dependent GSSG transport is inhibited by DNP-SG. Furthermore, canalicular transport of DNP-SG is sensitive to the sulfhydryl reagents NEM and DTNB [13]. Thus, whether DNP-SG transport system in the canalicular membrane is related to the low-K_m or high-K_m processes in the erythrocyte membrane remains to be elucidated. Western blot analysis with antibodies against a protein with DNP-SG-ATPase activity isolated from human erythrocyte membranes showed that the enzyme is expressed in various other human tissues including liver [18].

In conclusion, we suggest the existence of a red cell membrane protein capable of high-affinity transport of both DNP-SG and GSSG. This transporter has a higher affinity for the glutathione conjugate studied than for GSSG and does not transport GSH.

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