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The role of thiols in ATP-dependent transport of *S*-(2,4-dinitrophenyl) glutathione by rat liver plasma membrane vesicles

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Abstract

The effect of thiol/disulfide exchange on ATP-dependent *S*-(2,4-dinitrophenyl)glutathione (GS-DNP) transport was studied in sodium nitrate treated rat liver plasma membrane vesicles. Transport followed Michaelis-Menten kinetics with an apparent K_m of 9.6 μM for GS-DNP and 124 μM for ATP. 5,5′-Dithiobis(2-nitrobenzoate) (DTNB) and *N*-ethylmaleimide (NEM) efficiently inactivated GS-DNP transport activity in a dose- and time-dependent manner. Half-maximal inactivation occurred in 10 min at 40 μM for DTNB and 550 μM for NEM. Inactivation by DTNB was reversed by dithiothreitol. *S*-(*N*-Ethyl)maleimyl glutathione and/or ATP-Mg^{2+}, but neither *S*-(*N*-ethyl)maleimyl cysteinylglycine nor oxidized glutathione could protect transport activity from inactivation by NEM or cystamine. These results suggest that reactive thiols are located near the active site of the transporter and that *S*-alkyl and the γ-glutamyl residues of glutathione are important for protection. Biological disulfides which were tested included cystine, oxidized glutathione, oxidized Coenzyme-A, oxidized lipoic acid, and oxidized lipoamide; cystamine was the most potent reversible inactivator. Molecular oxygen also inactivated transport activity, which was recovered on addition of dithiothreitol, suggesting intramolecular disulfide formation by vicinal thiols. We interpret these results to indicate that the ATP-dependent GS-DNP transporter contains two or more thiols which are necessary for the maintenance of transport activity. The reversible inactivation of the activity by biological disulfides suggests that the transporter may be regulated by thiol/disulfide exchange in vivo.

Keywords: Glutathione conjugate; ATP-dependent transport; Sulfhydryl modification; (Rat liver)

1. Introduction

Glutathione (GSH)[1] has many important functions in cells: maintenance of the cellular redox state providing protection from oxidative stress, metabolic regulation of protein function by thiol/disulfide exchange, biosynthesis of physiologically active substances, and detoxification of nucleophilic compounds, including heavy metals [1–8]. Previous investigations demonstrated that GS-conjugates are eliminated from cells by an ATP-dependent process in various tissues and organisms, including yeast and plants [9–19]. This transporter effluxes not only GS-conjugates, but also bromosulfophthalein, glucuronide- or taurine-conjugates and other divalent anions [20–23]. In hepatocytes, the transporter is located in the bile canalicular domain of the plasma membrane and is important in transporting various organic anions out of the cell. TR^- rats lack this function in liver, but not in erythrocytes [10,21,24–26], indicating that there may be several gene products or that there are tissue-specific differences in regulation or gene expression.

Recently, two candidates have been proposed as GS-DNP transporters in mammalian cells. The first is the MRP 1 (multidrug resistance-associated protein) gene product, a 190 kDa protein which is a member of the ATP-binding

Abbreviations: NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); NTCB, 2-nitrothiocyanobenzoate; DTT, dithiothreitol; GS-DNP, *S*-(2,4-dinitrophenyl)glutathione; GS-NEM, *S*-(*N*-ethyl)maleimyl glutathione; Cys(Gly)-NEM, *S*-(*N*-ethyl)maleimyl cysteinylglycine; Cys-NEM, *S*-(*N*-ethyl)maleimyl cysteine; GSH, γ-glutamylcysteinylglycine (glutathione); GSSG, oxidized form of glutathione; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; AMPCPP, adenosine 5′-[α,β-methylene]triphosphate; AMPPCP, adenosine 5′-β,γ-methylene-triphosphate; NO_3LMVs, sodium nitrate treated liver plasma membrane vesicles; EDTA, ethylenediaminetetraacetic acid; MDR, multidrug resistance; MRP1, multidrug resistance-associated protein.

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cassette (ABC-type) transport family of proteins [27]. This protein has been identified as a glutathione *S*-conjugate transporter in several tumor cell lines exhibiting multidrug resistance in the absence of *MDR1* overexpression and in normal cells following transfection with the *MRP1* gene and overexpression of the MRP1 protein [27–29]. However, as determined by Northern blot analysis, MRP1 expression in normal human liver was not detected [30]. Barely detectable expression was observed by an RNAse protection assay [31]. A second candidate transporter was purified by ATP and GS-DNP affinity chromatography from liver plasma membrane detergent extracts by Zimniak and colleagues [32,33]. This protein has an apparent molecular mass of 90 kDa and does not appear to be an ABC-type protein.

In an effort to characterize structural requirements of the transporter which are necessary for its activity, we initiated studies into the effects of reversible and irreversible sulfhydryl modification reagents on bile canalicular ATP-dependent GS-DNP transport.

2. Materials and methods

2.1. Plasma membrane vesicle preparation from rat livers

Liver plasma membrane vesicles were prepared from male Wistar rats (200–300 g), using the third pellet as described by Ray [34] followed by sodium nitrate treatment. Briefly, dissected liver from anesthetized rats was perfused with ice-cold saline to remove erythrocytes, and scraped on ice while adding 10 mg/g liver tissue of phenylmethylsulfonyl fluoride as a proteinase inhibitor. The scraped liver tissue was homogenized with a Dounce loose pestle (25 strokes) and diluted 100-times the wet weight with cold 1 mM sodium bicarbonate containing 0.5 mM $CaCl_2$ (pH 7.8), and allowed to stand for 10 min. The diluted homogenate was filtered through six layers of cheesecloth and centrifuged at 3000 rpm for 30 min without brake at 4°C using a Beckman JA14 rotor. This first pellet was saved and gently homogenized (five strokes). The suspension was diluted to half the previous volume (50-times) and centrifuged at 2500 rpm for 20 min at 4°C. Only the fluffy pellet was collected (the second pellet), homogenized again with five gentle strokes, and centrifuged as above. The fluffy pellet was saved and centrifuged at 25 000 rpm for 30 min at 4°C using a Beckman 45 Ti rotor. The resulting pellet (designated as the third pellet) was resuspended at 1 mg protein/ml in 20 mM Tris-Hepes/10% glycerol (THG) buffer (pH 7.4), containing 0.5 M $NaNO_3$, 1 mM EDTA and 1 mM benzamidine. The mixture was gently stirred at room temperature for 60 min and ultracentrifuged as above. The pellet was washed (at least 100-times dilution) twice with THG buffer and centrifuged as above. The pellet designated as sodium nitrate treated liver membrane vesicles (NO_3LMVs) was resuspended in a minimum volume of THG buffer and stored at −80°C until used.

2.2. Transport studies

ATP-dependent transport of GS-DNP into NO_3LMVs was measured by a rapid filtration method as described, previously [22,23]. GS-DNP uptake in the absence of ATP was subtracted from that in the presence of ATP. Unless otherwise stated, the standard reaction mixture contained NO_3LMVs (1.5–2.5 mg/ml), 6 μM [*glycine*-2-^{3}H]GS-DNP, 4 mM $MgSO_4$ ± 4 mM ATP/Tris and an ATP-regenerating system (10 mM creatine phosphate and 25 U/ml creatine kinase) in THG buffer (pH 7.4) at 37°C. Transport was started by adding NO_3LMVs and stopped at the appropriate time by diluting into 1 ml of ice-cold THG buffer. The diluted mixture was passed through a Whatman GF/C glass filter (1.2 μm pore size); membranes were washed with 6 ml of ice-cold THG buffer. Radioactivity on the dried filter was soaked in Ready Safe™ (Beckman) and measured using a liquid scintillation counter (Beckman LS-1801).

2.3. Modification of reactive thiols by various sulfhydryl reagents and oxygen species

NO_3LMVs (2–4 mg/ml) were incubated at 25°C for various times in various concentrations of sulfhydryl reagents and oxygen species in 0.3–0.6 ml degassed THG buffer containing 0.5 mM EDTA. The incubation buffer was prepared at pH 7.4 for dithiobis(2-nitrobenzoate) (DTNB) and 2-nitrothiocyanobenzoate (NTCB), pH 7.2 for *N*-ethylmaleimide (NEM) and pH 8.0 for biological disulfides. The pH of all reagents was adjusted with Tris base just before use. Reactions were started by adding sulfhydryl reagents to the NO_3LMVs and stopped by diluting the mixture with 25 ml of ice-cold degassed THG buffer containing 1 mM EDTA. The diluted mixture was centrifuged at 25 000 rpm (Beckman 60 TI rotor) for 30 min. After washing once with 25 ml of THG buffer, the pellet was resuspended in 1 ml buffer and microfuged at 14 000 rpm for 10 min. The resulting pellet was resuspended to 150–200 μl of THG buffer (pH 7.4) and immediately used for transport studies. Some mixtures were further treated with 10 mM DTT for 30 min at 25°C before the dilution-centrifugation procedure. In case of longer incubation with GSSG or cystine for 16 h at 4°C, 0.5 ml of NO_3LMVs (4.0 mg/ml) were dialyzed against 500 ml of degassed THG buffer, 1 mM EDTA (pH 7.4) containing 0.5 mM GSSG and 1 mM cystine. The resulting mixture was extensively dialyzed in THG buffer containing 1 mM EDTA to remove excess unreacted sulfhydryl reagent changing buffer three times every 3 h. Treatment with 10 mM DTT was undertaken by dialysis with THG buffer containing 1 mM EDTA and DTT for 3 h before extensive dialysis.

Oxidation of NO_3LMVs (2.0 mg/ml) by oxygen species

was performed in 0.6 ml degassed THG buffer (pH 7.4) containing 50 μM $CuSO_4$, for 20 min. We used hydrogen peroxide (H_2O_2) alone or with catalase (56 U/ml) to generate molecular oxygen. The reaction was started by adding H_2O_2 at room temperature. After 20 min, the mixture was incubated with or without 10 mM DTT for an additional 30 min, and then subjected to the dilution-centrifugation procedure as described. To calculate H_2O_2 concentration, the molar extinction coefficient (43.6 M^{-1} cm^{-1} at 240 nm) for hydrogen peroxide was used [35].

2.4. Protection of reactive thiols from NEM and cystamine

NO_3LMVs (2.2 mg/ml) were preincubated at 25°C with 10 mM of various protectants in 0.6 ml degassed THG buffer containing 0.5 mM EDTA (pH 7.2), and 10 min later, the sulfhydryl reaction was started by adding NEM (0.4 mM); 60 min later, it was stopped by diluting the mixture into 25 ml of ice-cold THG buffer and centrifuging as described (vide supra).

2.5. Enzyme and protein assays

Mg^{2+}-ATPase, Na^+,K^+-ATPase, leucine aminopeptidase (LAP) and γ-glutamyltranspeptidase (γ-GTP) activity served as marker enzymes and were assayed [36–38]. Protein concentration was measured by the method of Bradford [39], using bovine serum albumin as a standard.

2.6. Chemicals and biochemicals

[*glycine*-2-^{3}H]Glutathione (43.8 Ci/mmol) was purchased from Du Pont-New England Nuclear. Other chemicals and biochemicals of reagent grade were obtained from Sigma. Sulfhydryl reagents were prepared immediately prior to use. All other reagents were prepared as greater than 10 × concentrated stock solutions, and the pH was adjusted before dilution.

[^{3}H]GS-DNP was synthesized from [*glycine*-2-^{3}H]glutathione and 1-chloro-2,4-dinitrobenzene enzymatically using glutathione *S*-transferase as described [9]. The recovery of radioactive product was about 80% and purity was greater than 95% as assessed by HPLC. *S*-(*N*-ethyl)maleimyl conjugates of GSH, cysteinylglycine (Cys(Gly)) and cysteine (Cys) were non-enzymatically synthesized by co-incubation with reduced GSH and NEM, Cys(Gly) and NEM, and Cys and NEM, respectively, as described in [18]. The extent of conjugation was monitored using DTNB to detect the amount of free sulfydryl remaining in the reaction mixture [40]. Reverse-phase HPLC was used to check the purity of all conjugates.

3. Results

3.1. Characterization of NO_3 LMVs

Canalicular marker enzymes, LAP, γ-GTP and Mg^{2+}-ATPase, were enriched 3–6-fold in crude liver plasma membrane (Ray's third pellet) with respect to homogenate (Table 1). Enrichment of Na^+,K^+-ATPase, a sinusoidal marker enzyme, was less than half. Mg^{2+}-ATPase/Na^+,K^+-ATPase activity ratio increased 7.2-fold from 1.2 to 8.6. Cytochrome oxidase was slightly contaminating, but lactate dehydrogenase was not detected even after sonication (data not shown). These data agree with prior characterization of canalicular membranes [41]. Although Ray's third pellet is still crude, it contained ATP-dependent GS-DNP transport activity (2.51 ± 2.03 pmol/mg per min). To obtain higher activity, we tried to remove superficially or loosely bound proteins by treatment with chaotropic reagents including NaSCN, KI, $NaNO_3$ and $NaClO_4$. Treatment with 0.5 M $NaNO_3$ for 60 min was the best, resulting in 8-fold higher specific activity and 3-fold increase in total activity, which might be responsible for removal of endogenous inhibitors against transport system. Transport activity was retained in NO_3LMVs, suggesting that the GS-DNP transporter is associated tightly with the plasma membrane. Protein recovery as NO_3LMVs was 97.7 ± 25.6 mg (about 1.8% of total homogenate protein) from 40 g of rat liver.

ATP-mediated GS-DNP uptake increased in a time- and dose-dependent manner showing linear uptake for 4 min (Fig. 1a). Transport was saturable (Fig. 1b and Fig. 1c).

Table 1
Protein recovery and activity of ATP-dependent GS-DNP transport and marker enzymes

	Homogenate	3rd pellet	3rd pellet treated with $NaNO_3$
Protein from 40 g wet liver	5.48 ± 1.1 g	231 ±49.6 mg	97.7 ± 25.6 mg
Transport activity (pmol/mg per min)		2.53 ± 2.03	19.6 ± 5.63
Total activity (pmol/min)		≈ 580	≈ 1920
	nmol/min per mg protein		
Mg^{2+}-ATPase	151 ±44	483 ±32	591 ±76
Na^+,K^+-ATPase	130 ±26	56 ± 3.5	43 ± 1.9
Leucine aminopeptidase	16 ± 3.1	50 ± 4.0	105 ± 6.1
γ-Glutamyltransferase	0.9 ± 0.29	5.4 ± 0.4	19.3 ± 6.7

All values are measured as described in Materials and methods and represent the means ± S.D. from four separate experiments.

The apparent K_m values were 9.6 μM and 124 μM for GS-DNP and ATP, respectively, which are similar to the previous values reported by Kobayashi et al. [12]. As shown in Table 2, hydrolyzable phosphorus in the γ-position was required for GS-DNP transport (ADP, AMP and AMPPCP were 19, 9 and 5% of control, respectively) in a vanadate-inhibitable fashion (37% of control in 0.1 mM orthovanadate). ATP was specifically required, since AMPCPP (37%), GTP (16%), UTP (17%) and CTP (32%) were poor energy sources for GS-DNP transport as previously demonstrated [9,12,18,19,23]. GS-DNP and GS-NEM (0.1 mM each) inhibited ATP-dependent [^{3}H]GS-DNP uptake to 29% and 74%, respectively as compared to controls. Cys(Gly)-NEM (93%), Cys-NEM (105%), GSSG (87%) and GSH (105%) were not good inhibitors, possibly due to lack of an *S*-alkyl moiety at cysteine and/or γ-glutamyl residue of GSH. 10 mM GS-NEM completely inhibited transport activity, whereas 10 mM GSSG inhibited only 50%. Taurocholate and daunomycin were poor inhibitors, confirming that the GS-DNP transporter is distinct from those responsible for taurocholate and daunomycin transport [9,12,21,23,42–44]. ATP-dependent taurocholate transport was also found in NO_3LMVs (41.3 pmol/mg per min at 4.8 μM taurocholate) and was as high as in purified canalicular membrane vesicles [42–44]. NO_3LMVs also contained immunoreactive p-glycoprotein which reacted with antibody C219 (purchased from CENTOCOR, Malvern, PA) by Western blotting (data not shown) [45].

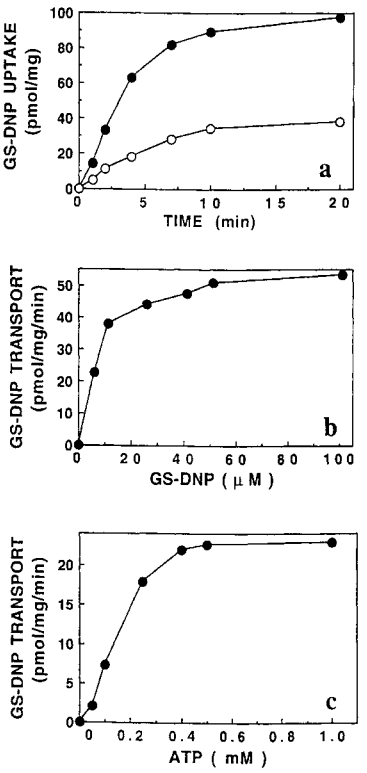


Fig. 1. Time course of GS-DNP uptake and effect of GS-DNP/ATP on ATP dependent GS-DNP transport by sodium nitrate treated liver membrane vesicles (NO_3LMVs) (a) NO_3LMVs (560 μg) were incubated for appropriate time at 37°C with 5 μM [^{3}H]GS-DNP in the presence (●) or absence (○) of 4 mM ATP/Tris in 280 μl of buffer containing 20 mM Tris/Hepes, 10% glycerol, 4 mM $MgSO_4$, 10 mM phosphocreatine and 100 mg/ml creatine kinase (pH 7.4) (buffer A). (b) NO_3LMVs (560 μg) were incubated at 37°C for 4 min with various concentration of [^{3}H]GS-DNP in 280 μl of buffer A in the presence or absence of 4 mM ATP/Tris. (c) NO_3LMVs (560 μg) were incubated at 37°C for 4 min with various concentration of ATP/Tris and 6 μM [^{3}H]GS-DNP in 280 μl of buffer A. Vesicle-associated GS-DNP uptake and ATP-stimulated GS-DNP transport were determined as described under Materials and methods. All values represent the mean of duplicates in two separate experiments.

Table 2
Effects of various nucleotides, GSH derivatives, vanadate, taurocholate and daunomycin on ATP-dependent GS-DNP transport in $NaNO_3$-treated plasma membrane vesicles

		Relative transport activity [c]
Nucleotide specificity [a]	(mM)	(%)
ATP	5.0	100
GTP	5.0	16
UTP	5.0	17
CTP	5.0	32
ADP	5.0	19
AMP	5.0	9
AMPPCP	5.0	5
AMPCPP	5.0	37
Inhibitory effect [b]		
Control (22.3 pmol/mg per min)		100
GSH	0.1	142 (104, 181) [d]
GSSG	0.1	87 (93, 81) [d]
	10.0	50
GS-DNP	0.1	29
GS-NEM	0.1	74 (80, 71) [d]
	5.0	35
	10.0	0
Cys(Gly)-NEM	0.1	93 (92, 94) [d]
Cys-NEM	0.1	105
	(μM)	(%)
Orthovanadate	100	37
Taurocholate	50	91
Daunomycin	50	109

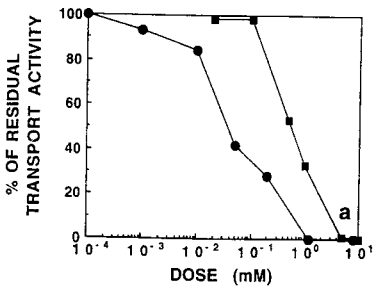
[a] NO_3LMVs (2.0 mg/ml) were incubated at 37°C for 2 min in THG buffer (pH 7.4), 5 mM $MgSO_4$, 6 μM [^{3}H]GS-DNP, ±5 mM various nucleotides in the absence of creatine kinase and phosphocreatine.
[b] In the presence of the indicated substrates, ATP-dependent GS-DNP transport activity was measured under standard conditions as described in Materials and Methods, except for 88 μM [^{3}H]GS-DNP.
[c] Values represent mean of % control activities from duplicate determinations.
[d] Indicates the values from two separate preparations shown in parentheses.

3.2. *Effect of chemical sulfhydryl reagents and oxygen species on ATP-dependent GS-DNP transport*

ATP-dependent GS-DNP transport was inactivated by DTNB and NEM in a dose- and time-dependent manner (Fig. 2a and Fig. 2b), with half-maximal inactivation con-

centrations at 10 min of 40 μM and 550 μM, respectively. Complete inactivation was accomplished at 1.2 mM DTNB and 5 mM NEM in 10 min. Inactivation by DTNB was reversible on addition of 10 mM DTT; reactivation was 75% and 95% of the original activity in 10 and 60 min, respectively. These results suggest that the transporter may contain reactive thiols, modification of which diminishes transport ability. Similarly, NTCB, which creates the S-CN moiety, effectively decreased transport activity to 21%. Complete recovery of activity occurred following treatment with DTT (Table 3).

Molecular oxygen has a bi-directional oxidative function. On reaction with thiol as an oxygen donor, it produces irreversible sulfenic, sulfinic or sulfonic (cysteic) residues, whereas, if reacted as a dehydrant, it creates reversible intra- or intersubunit disulfides with adjacent thiols [46,47]. Molecular oxygen generated from H_2O_2 with catalase (56 U/ml) inactivated transport activity to 26% and 0.4% using 4 and 6 mM H_2O_2, respectively (Table 3). The oxidized transporter was partly reactivated with DTT, which suggests that a disulfide linkage had been created. Low recovery of activity implies that some



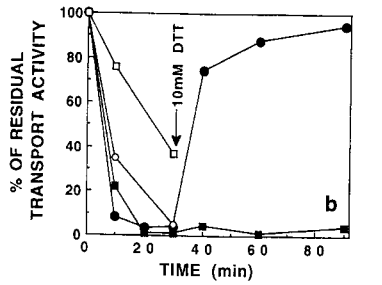


Fig. 2. Inactivation of ATP-dependent GS-DNP transport by sulfhydryl reagents and effect of dithiothreitol (DTT). (a) Sodium nitrate treated liver membrane vesicles (NO_3LMVs) (2 mg/ml) were incubated with various concentration of DTNB (●) and NEM (■) at pH 7.4 and 7.2, respectively, for 10 min in 20 mM Tris/Hepes, 10% glycerol (THG) buffer containing 0.5 mM EDTA at room temperature. (b) NO_3LMVs (4.3 mg/ml) were incubated with 0.1 mM (○), 0.4 mM (●) DTNB and 0.4 mM (□), 1.6 mM (■) NEM for indicated time in 20 mM THG buffer containing 0.5 mM EDTA at room temperature. The pH of reaction mixture was 7.4 for DTNB and 7.2 for NEM. 10 mM DTT was added at 30 min to the mixture of both 0.4 mM DTNB and 1.6 mM NEM. All reactions were stopped by dilution and centrifugation method and ATP-dependent GS-DNP transport was assayed at 37°C for 4 min. as described in Materials and methods. All values represent the mean of duplicates in two separate experiments.

Table 3
Modification of reactive thiols of GS-DNP transporter by chemical sulfhydryl reagents, biological disulfides and oxygen species

Compounds		Time	Residual ATP-dependent GS-DNP transport activity (%) [a]	
	mM	(min)		DTT treated
Chemical reagents				
DTNB	1.0	10	5.2	105
NTCB	1.0	10	21 (31, 13) [b]	109 (100, 118) [b]
NEM	1.0	10	20	15
Biological disulfides				
GSSG	0.5	16 h	41	96
	1.5	90	82	
	10.0	60	86	
Cystine	0.2	30	98	
	1.0	16 h	43	123
Cystamine	0.5	60	45	
	2.0	60	19	70
CoASSCoA	0.6	90	79	
Lipoic acid	0.5	60	96	
(oxidized)	2.0	60	80	
Lipoamide	0.5	60	100	
(oxidized)	2.0	60	100	
Oxygen species				
H_2O_2	4	20	90	102
	6	20	83	84
H_2O_2 /catalase	4	20	26	69
	6	20	0.4	20

Methods of sulfhydryl modification, subsequent DTT treatment and ATP-dependent GS-DNP transport assay were described in Materials and methods.The incubation time for transport was 4 min.

[a] Values represent mean of % control activities from duplicate determinations.

[b] Indicates the values from two separate preparations shown in parentheses.

reactive thiols were irreversibly oxidized to sulfonic acid or that oxidation occurred on other amino acids [48] essential for transport. These results imply that the GS-DNP transporter contains at least two reactive thiols. Hydrogen peroxide alone slightly inactivated the transport activity (4 and 6 mM H_2O_2 were 90 and 83% of controls, respectively), suggesting that oxygen is a more efficient oxidant of the GS-DNP transporter than is hydrogen peroxide alone.

3.3. Protection of reactive dithiols of the transporter

If reactive thiols are located near the active site, transport activity should be protected from thiol oxidation in the presence of substrates. We determined whether GS-NEM, GSSG and ATP could protect the GS-DNP transporter against sulfhydryl modification. ATP/Mg^{2+} protected the transporter from NEM inactivation (90%), but neither ATP (9%) nor Mg^{2+} (13%) protected (Table 4), suggesting that a conformational change of ATP by Mg^{2+} [49] is critical for binding to the catalytic site in addition to protecting reactive thiols. GS-NEM alone or with ATP/Mg^{2+} protected transport activity against NEM and

Table 4
Protection of GS-DNP transport activity from NEM and cystamine usingspecified substrates

	Restored activity (%)
Control (15 pmol/mg per min)	100
0.4 mM NEM, no addition	17 (13,20) [a]
+ ATP	9
+ $MgSO_4$	13
+ ATP + $MgSO_4$	90 (83,97) [a]
+ GS-NEM	108
+ GS-NEM + ATP + $MgSO_4$	102
+ Cys(Gly)-NEM	8
+ GSSG	12 (9,15) [a]
+ GSSG + ATP	27
+ GSSG + ATP + $MgSO_4$	57
+ 0.5 M KCl	8
5 mM cystamine, no addition	11
+ ATP + $MgSO_4$	99

NO_3LMVs (2.2 mg/ml) were preincubated for 10 min with various transporter substrates at pH 7.4. Sulfhydryl modifications were started by adding NEM or cystamine and reactions were stopped after 60 min as described in Materials and methods. Transport was performed as described in Table 2. The incubation time was 4 min. All protective agents added are 10 mM except for KCl. Values represent mean of % control activities from duplicate determinations.

[a] Indicates the values from two separate preparations shown in parentheses.

preserved activity 108% and 102%, as compared with controls. However, Cys(Gly)-NEM, which lacks the γ-glutamyl residue of GS-NEM, did not protect (8% protection). These results indicate that reactive dithiols and the γ-glutamyl residue of GSH conjugates may interact. 0.5 M KCl alone did not protect transport activity. Surprisingly, GSSG, which has been thought to be a substrate of the GS-DNP transporter [9,12,13], did not protect transport activity, with or without ATP (15% or 27%, respectively). This result is consistent with the weak ability of GSSG to inhibit GS-DNP transport (Table 2) and suggests that the weak binding of GSSG to the transporter may be due to lack of an *S*-alkyl moiety of GSH. Coincubation of GSSG and ATP/Mg^{2+} showed 57% protection from inactivation by NEM.

3.4. Effect of biological disulfides on ATP-dependent GS-DNP transport

Reactive thiol residues of a protein can form mixed disulfides with endogenous biological disulfides in vivo resulting in altered function [2,3]. Several biological disulfides were added to NO_3LMVs as sulfhydryl reagents. Treatment with 0.5 and 2.0 mM cystamine for 60 min reversibly decreased GS-DNP transport activity to 45% and 19% of control, respectively (Table 3). Among the biological disulfides tested, cystamine was the best inhibitor. Incubation for up to 90 min with GSSG, Coenzyme A (oxidized form) and lipoic acid (oxidized form) moderately inactivated GS-DNP transport to approx. 82%, 79% and approx. 80% of controls, respectively, and both cystine and lipoamide (oxidized form) showed no effect on transport activity. However, prolonged exposure (16 h) to 0.5 mM GSSG and 1 mM cystine significantly reduced transport activity to 41% and 43%, respectively; subsequent treatment with 10 mM DTT resulted in total recovery of activity. These findings suggest that biological disulfides may be potent inactivators of ATP-dependent GS-DNP transport in vivo, most likely due to their ability to produce protein-mixed disulfides.

4. Discussion

In this report, we characterized an ATP-dependent GS-DNP transport activity in NO_3LMVs which has requirements similar to those described for the ATP-dependent GS-DNP transporter in the canalicular membrane [11–13,23,50]. The increase in the specific activity of transport in the NO_3LMV preparation correlates with the enrichment of canalicular enzymes, suggesting that the transporter resides in the canalicular membrane. ATP-dependent GS-DNP transport in NO_3LMVs requires hydrolyzable γ-phosphate and steric configuration of ATP because other nucleotides, including ATP analogs, result in poor transport activity [9,12,13,18,19]. Lack of an *S*-alkyl moiety (GSSG) and the γ-glutamyl residue (Cys(Gly)-NEM, Cys-NEM) of GSH results in little inhibition of GS-DNP transport, which was confirmed by protection study of thiols against NEM (Table 4). Therefore, the binding affinity of GSSG [9,12,13,18], Cys(Gly)-NEM, and Cys-NEM may be lower than that of GS-DNP or GS-NEM.

The results of thiol modification suggest that the GS-DNP transporter has thiols which are sensitive to sulfhydryl reagents and can be modified in a dose- and time-dependent manner (Fig. 2a and Fig. 2b and Table 3). Inactivation by DTNB, NTCB and biological disulfides was reversed on addition of DTT. In addition, oxygen gas flushing (1 L/min for 1 min) of NO_3LMV suspensions also decreased the transport activity by about 50%, whereas nitrogen gas flushing did not (data not shown). Reversible inactivation with molecular oxygen indicates that the transporter has two or more reactive thiols which can form a disulfide linkage (Table 3). Kondo et al. [14] and Akerboom et al. [51] reported that ATP-dependent GS-DNP transport activity was increased by DTT or GSH treatment, which might be due to reactivation of the transporter which had been already oxidized by atmospheric oxygen (autooxidation) during vesicle preparation. This evidence suggests that prevention of autooxidation of the GS-DNP transporter should minimize loss of the activity during preparation of canalicular membrane vesicles and purification of the transporter. In this study, all buffers containing EDTA were degassed by helium bubbling and excess of sulfhydryl reagent was carefully removed prior to GS-DNP transport assay. Creatine kinase in the assay mixture is also very

sensitive to such reagents [52]. GS-DNP (GSSG) transport activity in canalicular membrane vesicles [13] including NO_3LMVs is more sensitive to inactivation by sulfhydryl reagents than is erythrocyte activity [14,15,51,53], which may reflect cell-specific differences in gene expression. This is supported by studies using TR^- mutant rats which lack hepatocyte ATP-dependent GS-DNP transport activity but retain similar transport in erythrocytes [25].

Lack of ATP/Mg^{2+} configuration and absence of an *S*-alkyl moiety or γ-glutamyl residue of GSH resulted in poor protection against NEM inactivation, suggesting that reactive thiols are important for interaction with substrates which have high binding affinity (Table 4). These data also suggest that the binding sites for ATP/Mg^{2+} and GS-DNP are close to each other, since ATP/Mg^{2+} and GS-DNP protect independently, not additively, against the same reactive thiols.

There is evidence to suggest that cysteine-thiols are important for transport activity. The reactive Cys^{384} of the mannitol transporter must be phosphorylated for uptake of mannitol into the cell. Modification of Cys^{384} causes the transporter to lose its activity [54]. For the proline carrier of *E. coli*, modification of cysteine by NEM inhibits transport activity. Additionally, substrates can protect this cysteine from modification and preserve activity [55]. Furthermore, several studies utilizing NEM, NBD-Cl and $HgCl_2$ suggest that cysteines are important for both the transport [56] and ATPase activities of p-glycoprotein [57–59], and that ATP can protect against inactivation by NEM [58]. More important is the location of a conserved cysteine residue in the 'Walker homology A domain' of human MRP1, MDR1 and MDR2 and all hamster p-glycoproteins (personal observation) [57]. Conservation of this cysteine suggests that it may be critical for transport and ATPase activities. However, other studies indicate that cysteines are not essential for activity, but may be important for protein stability or folding. Loo and Clarke created a cysteine-less mutant of human MDR 1 and transfected the gene into NIH 3T3 cells [60]. Mutation of all cysteines to alanines led to a slight decrease in drug resistance, as determined by cell viability. They concluded that cysteine residues are not essential for MDR 1 activity, but are important for protein stability, because the cysteine-less protein was glycosylated more slowly and had a shorter half-life. However, because they did not measure the functional activity of the protein, i.e., drug efflux or drug-stimulatable ATPase activity, it is not possible to compare their results directly with those of Al-Shawi et al. [57–59].

The identification of two different gene products as ATP-dependent GS-conjugate efflux pumps [27–29,32,33] indicates that there is likely to be more than one ATP-dependent, canalicular GS-conjugate transporter. The present study shows that thiol modification of the transporter diminished transport activity. However, whether these thiols are essential for substrate (GS-DNP) transport and/or binding remains to be elucidated.

Recently, Feng and Forgac proposed an endogenous activation/ inactivation model of clathrin-coated vesicle H^+-ATPase by switching disulfide linkages in which Cys^{532}-Cys^{277} is the active form and Cys^{532}-Cys^{254} is the inactive form [61]. It will be of interest to see whether the GS-DNP transporter can be regulated by this type of switching or whether other biological factors cause thiol/disulfide exchange. There are many potential regulating factors for thiols, such as altered cytoplasmic redox state caused by exogenous signals including oxidative stress, thiol modification by biological disulfides, and enzymatic disulfide exchange by protein disulfide isomerase or thioltransferase [1–3]. Biological disulfides and oxygen species decrease ATP-dependent GS-DNP transport activity in a manner similar to their effects on other cytosolic enzymes. Among biological disulfides, cystamine, a metabolic intermediate of taurine synthesis from cysteine, was the best inhibitor of the GS-DNP transport activity. In addition, reactive oxygen is a potent inactivator that is generated during oxidative stress and as a result of normal cellular aerobic metabolism [62]. These observations suggest that functional regulation of ATP-dependent GS-DNP transport by thiol/disulfide exchange may occur in vivo.

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