Inhibition of Mrp2- and Ycf1p-mediated transport by reducing agents: evidence for GSH transport on rat Mrp2

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Abstract

Mammalian Mrp2 and its yeast orthologue, Ycf1p, mediate the ATP-dependent cellular export of a variety of organic anions. Ycf1p also appears to transport the endogenous tripeptide glutathione (GSH), whereas no ATP-dependent GSH transport has been detected in Mrp2-containing mammalian plasma membrane vesicles. Because GSH uptake measurements in isolated membrane vesicles are normally carried out in the presence of 5–10 mM dithiothreitol (DTT) to maintain the tripeptide in the reduced form, the present study examined the effects of DTT and other sulfhydryl-reducing agents on Ycf1p- and Mrp2-mediated transport activity. Uptake of S-dinitrophenyl glutathione (DNP-SG), a prototypic substrate of both proteins, was measured in Ycf1p-containing *Saccharomyces cerevisiae* vacuolar membrane vesicles and in Mrp2-containing rat liver canalicular plasma membrane vesicles. Uptake was inhibited in both vesicle systems in a concentration-dependent manner by DTT, dithioerythritol, and β-mercaptoethanol, with concentrations of 10 mM inhibiting by ∼40%. DTT's inhibition of DNP-SG transport was noncompetitive. In contrast, ATP-dependent transport of [^{3}H]taurocholate, a substrate for yeast Bat1p and mammalian Bsep bile acid transporters, was not significantly affected by DTT. DTT also inhibited the ATP-dependent uptake of GSH by Ycf1p. As the DTT concentration in incubation solutions containing rat liver canalicular plasma membrane vesicles was gradually decreased, ATP-dependent GSH transport was now detected. These results demonstrate that Ycf1p and Mrp2 are inhibited by concentrations of reducing agents that are normally employed in studies of GSH transport. When this inhibition was partially relieved, ATP-dependent GSH transport was detected in rat liver canalicular plasma membranes, indicating that both Mrp2 and Ycf1p are able to transport GSH by an ATP-dependent mechanism. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Mrp2; YCF1; Glutathione; Membrane transport; Dithiothreitol

1. Introduction

Reduced glutathione (GSH) is the major cellular reductant, and plays an important role in protection against toxic chemicals [1,2]. GSH is synthesized in the cell cytosol, but is eventually exported across the plasma membrane for degradation in the extracellular space [3–5]. Although GSH-specific plasma membrane transporters have not been identified, GSH is a

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substrate for the organic anion transporting polypeptides Oatp1 and Oatp2 [6,7], the multidrug resistance-associated proteins Mrp1 and Mrp2 [8–21], and the yeast orthologue of these Mrp proteins, the vacuolar membrane transport pump Ycf1p [22,23]. Mammalian Mrp1 and Mrp2, and Ycf1p of the budding yeast *Saccharomyces cerevisiae* are ATP-binding cassette (ABC) proteins that export organic anions, including glutathione *S*-conjugates, concomitant with ATP hydrolysis. These proteins have a similar substrate specificity, although there appears to be a difference in their ability to transport GSH. GSH is a low-affinity ATP-dependent substrate for Ycf1p [22,23], whereas studies in mammalian Mrp1- or Mrp2-containing plasma membrane vesicles have failed to detect ATP-dependent GSH transport ([8], for review). The reason for this apparent discrepancy between the yeast and mammalian Mrp orthologues is unclear, but some possibilities have recently been described [8,20], including: (a) a relatively high nonspecific permeability of rat liver vesicles when compared with yeast secretory vesicles, which tends to dissipate solute gradients; (b) a high ATP hydrolysis rate in liver plasma membrane vesicles, which leads to the rapid depletion of the driving force for transport; and (c) a relatively low catalytic efficiency of GSH transport in rat liver membrane vesicles (i.e. a high K_m and only modest V_{max}).

The present study identified another factor that contributes to the inability to detect ATP-dependent GSH transport in liver membrane vesicles, namely the inhibitory effect of dithiothreitol (DTT), the reducing agent that is normally added to prevent GSH oxidation. Because all previous studies of GSH transport have utilized high concentrations of reducing agents, they probably underestimated GSH transport rates.

2. Experimental procedures

2.1. Materials

[*glycine*-2-³H]GSH (0.982 Ci/mmol) and [G-³H]-taurocholate (2.1 Ci/mmol) were purchased from DuPont NEN. [*glycine*-2-³H]*S*-Dinitrophenyl glutathione (DNP-SG) was synthesized as previously described [24]. Zymolyase-100T was purchased from

Seikagaku America (Rockville, MD, USA). All other chemicals and reagents were purchased from Sigma-Aldrich, Amersham, J.T. Baker, or Fisher. The pH of incubation solutions containing high concentrations of GSH or other anions was routinely adjusted to 7.5 immediately before use with Tris base.

2.2. Yeast strain and vacuole isolation

DTY165 (MATα ura3-52 his6 leu2-3,-112 his3-Δ200 trp1-901 lys2-801 suc2-Δ) yeast strain of *S. cerevisiae* was kindly provided by Dennis Thiele (University of Michigan) and grown in YPD medium as described by Li et al. [25,26]. Vacuolar membranes were prepared and characterized according to methods described previously [22,23]. Vesicles were resuspended and stored at −80°C in buffer containing 10 mM HEPES, pH 7.5, 250 mM sucrose, and 20 mM KCl.

2.3. Rat canalicular liver plasma membrane (cLPM) vesicles

Male Sprague–Dawley rats ($\sim$250 g) were obtained from Charles River Laboratories (Kingston, NY, USA) and fed Purina chow (formula 5001) ad libitum. The methods used to isolate and characterize cLPM were as described by Meier et al. [27] and previously performed in this laboratory [24,28]. The cLPM fraction contains vesicles that are both in the right-side-out ($\sim$80%) and inside-out ($\sim$20%) configuration [29]. For the inside-out vesicles, the extravesicular membrane face corresponds to the cytosolic surface in vivo. Vesicles were resuspended and stored at −80°C in buffer containing 10 mM HEPES, pH 7.5, 250 mM sucrose, and 20 mM KCl.

2.4. Transport assays

Transport was measured as uptake of radiolabeled substrate into vesicles collected by rapid filtration on Millipore 0.45 μm filter under vacuum, essentially as described previously [24]. Membrane vesicles were thawed by immersion in a 30°C water bath and diluted in transport buffer (10 mM HEPES, pH 7.5, 250 mM sucrose, 20 mM KCl, with an ATP regenerating system consisting of 10 mM phosphocreatine, 10 mM $MgCl_2$, 100 μg/ml creatine phosphokinase,

and either 5 mM Na_2ATP or 10 mM NaCl). Diluted vesicles were treated with 0.5 mM acivicin to inhibit resident γ-glutamyl transpeptidase enzyme, passed repeatedly through a 26 g needle ($\times 10$), and incubated at 30°C for 15 min prior to starting the transport reaction.

To minimize GSH oxidation in GSH transport assays without DTT, we reduced the amount of dissolved oxygen in our transport buffer by saturating the vacuum-degassed transport reaction solutions with N_2 for 30 min before initiating reaction incubations, and all incubations were done under nitrogen. An enzymatic conversion of glutathione disulfide (GSSG) to GSH was also utilized in which we added 0.125 mg of glutathione reductase and 0.25 mM NADPH to the transport reaction buffer. Transport was started by adding 20 µl of diluted membrane vesicles to 80 µl of incubation transport reaction buffer (with substrate) at 4°C or 30°C for timed intervals. Initial rates were determined where uptake was linear with time at each substrate concentration tested. Transport was quenched by adding 1 ml of ice-cold stop buffer (300 mM sucrose, 10 mM HEPES–Tris, pH 7.5, 20 mM KCl), and vesicles collected by applying 1 ml of quenched reaction solution to a prewetted filter under vacuum and washing the filter with an additional 4 ml of ice-cold stop buffer. Filters were collected, dissolved in 5 ml of
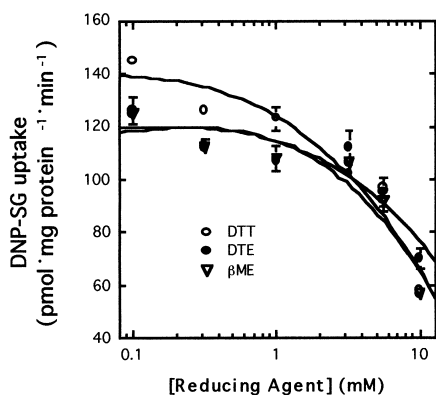

Fig. 1. Reducing agents inhibit the initial rate of ATP-dependent DNP-SG uptake in yeast vacuolar membrane vesicles. The initial rate of ATP-dependent uptake of 10 µM [³H]DNP-SG in *S. cerevisiae* DTY165 vacuolar plasma membrane vesicles was measured in the presence of increasing concentrations of DTT (○), DTE (●), or βME (▽). The data points represent the mean with standard errors of the differences between uptake measurements with 5 mM MgATP and those without ATP for each condition of at least three experiments.

Opti-Fluor (Packard Instrument, Meriden, CT, USA), and radiolabeled drug uptake measured by liquid scintillation counting. Controls for nonspecific binding of drug to filters and vesicles were performed by measuring retention of radiolabeled substrate on filters in the absence of vesicles and in vesicles incubated in transport buffer at 4°C for each time point.

2.5. Determination of GSH and GSSG concentrations

The concentrations of GSH and GSSG were measured in transport reaction buffers under conditions used to measure transport of GSH by an enzymatic procedure using glutathione reductase and 5,5′-dithio-bis(2-nitrobenzoic acid), either with or without derivatization of GSH with 1 µl of 2-vinylpyridine [30,31].

2.6. Statistical analysis

Kinetic data from experiments measuring initial rates of uptake of radiolabeled substrate in vacuolar vesicles were fit to the Michaelis–Menten equation by nonlinear least squares regression analysis using SigmaPlot 4.16. V_{max} and K_m values with standard errors were derived from these curves. Comparison of data measuring initial rates of uptake of [³H]DNP-SG or [³H]GSH in the presence and absence of inhibitors was performed by paired Student's *t*-test, and correlated to $P < 0.05$.

3. Results

3.1. Reducing agents inhibit ATP-dependent DNP-SG, but not taurocholate uptake in yeast vacuolar membrane vesicles

The vacuolar membrane of *S. cerevisiae* contains the ABC proteins Ycf1p, which can transport glutathione *S*-conjugates such as DNP-SG [23,25,26], and Bat1p, which can transport bile acids such as taurocholate [32]. Transport by both proteins is ATP-dependent. Examination of the initial rates of 10 µM [³H]DNP-SG uptake in *S. cerevisiae* vacuolar plasma membrane vesicles demonstrated that transport was inhibited by DTT (Fig. 1). A DTT concentration of 0.1 mM had no effect (data not shown), but trans-
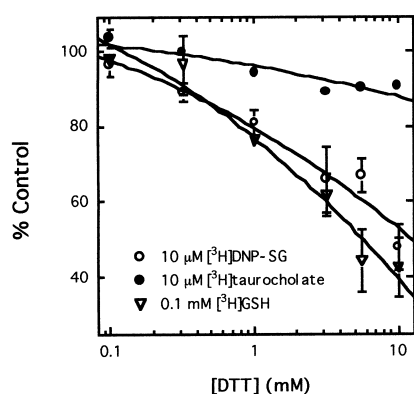
Fig. 2. Effects of DTT on the ATP-dependent uptake of DNP-SG, taurocholate, and GSH in yeast vacuolar membrane vesicles. The effect of increasing concentrations of DTT on the initial rate of ATP-dependent uptake of 10 μM [^{3}H]DNP-SG (○), 10 μM [^{3}H]taurocholate (●), and 0.1 mM [^{3}H]GSH (▽) was measured in vacuolar plasma membrane vesicles. The difference in uptake with 5 mM MgATP and without ATP was calculated to be the ATP-dependent transport. Data points represent the mean with standard errors of three experiments.

port was significantly reduced at DTT concentrations of 0.3 mM and higher (Fig. 1). Comparable results were obtained when DTT was replaced with two other sulfhydryl-reducing agents, dithioerythritol (DTE) or β-mercaptoethanol (βME) (Fig. 1). Each of these reducing agents showed statistically significant inhibition of ATP-dependent [^{3}H]DNP-SG transport at concentrations of 1 mM and higher.

ATP-dependent uptake of 0.1 mM [^{3}H]GSH in yeast vacuolar membrane vesicles was also inhibited by DTT (Fig. 2). Note that the effects of DTT on DNP-SG and GSH transport were quantitatively similar, supporting the conclusion that both compounds are substrates for Ycf1p. In contrast, DTT had only minimal effects on ATP-dependent 10 μM [^{3}H]taurocholate transport (Fig. 2), indicating that DTT's effects on Ycf1p-mediated transport are not due to a generalized effect on vesicle transport activity nor to a nonspecific change in membrane integrity.

3.2. Kinetics of DTT inhibition of ATP-dependent [^{3}H]DNP-SG transport in yeast vacuolar membrane vesicles

The mechanism of action of DTT on Ycf1p was examined by measuring the kinetics of [^{3}H]DNP-SG uptake with either 0, 1, 5, or 10 mM DTT (Fig. 3).

ATP-dependent uptake for each condition was fit to the Michaelis–Menten equation, and the data are presented as a Wolff–Augustinsson–Hofstee plot (Fig. 3, inset). The kinetic constants derived from this analysis are presented in Table 1. The Ycf1p apparent K_m for DNP-SG was 39 μM with a V_{max} of 9 nmol (mg protein)$^{-1}$ min^{-1}. The apparent affinity for DNP-SG was not significantly changed by DTT, whereas the V_{max} values were markedly reduced as the DTT concentration was increased. These data suggest that DTT inhibits Ycf1p-mediated transport of [^{3}H]DNP-SG in a noncompetitive manner, and therefore is presumably not affecting substrate binding.
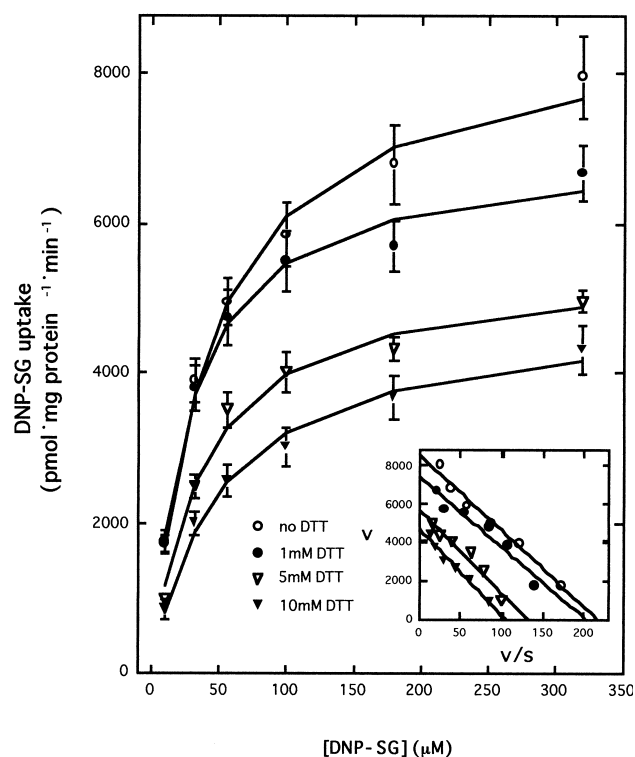
Because ATP is also a substrate for Ycf1p, we also

Fig. 3. DTT noncompetitively inhibits Ycf1p-mediated transport of DNP-SG in yeast vacuolar membrane vesicles. The effects of 0 (○), 1 (●), 5 (▽), and 10 mM DTT (▼) on the initial rate of ATP-dependent uptake of increasing concentrations of [^{3}H]DNP-SG were examined in yeast vacuolar membrane vesicles. The data points represent the mean with standard errors of at least three experiments. The data were fit by a least squares linear regression curve that followed the Michaelis–Menten equation ($P < 0.05$) to derive kinetic parameters. The data were further analyzed by a Woolf–Augustinsson–Hofstee plot to illustrate the kinetics of inhibition (inset).

examined the effect of 0, 1, 5, and 10 mM DTT on the ATP concentration-dependent initial rate of uptake of 10 μM [³H]DNP-SG (Fig. 4 and Table 1). The Ycf1p apparent K_m for ATP was 0.51 mM and the V_{max} was 0.36 nmol (mg protein)$^{-1}$ min^{-1} in the absence of DTT. DTT decreased the V_{max}, but had no significant effect on the K_m for MgATP (Fig. 4 and Table 1). These results indicate that DTT does not affect the transporter's relative affinity for ATP, but rather inhibits the transporter at some allosteric site distinct from both the ATP binding and substrate binding sites.

3.3. DTT also inhibits ATP-dependent transport of DNP-SG in rat cLPM vesicles

In support of the conclusions reached in the yeast vacuolar membranes, uptake of 10 μM [³H]DNP-SG by rat cLPM vesicles was also diminished by DTT, whereas [³H]taurocholate transport was not affected (Fig. 5), indicating that Mrp2 is inhibited by DTT, whereas the liver bile salt transporter (Bsep) is not. The ATP-independent components of DNP-SG and taurocholate uptake were small relative to the ATP-dependent components, and were only minimally affected by DTT.

Fig. 4. Effects of DTT on the kinetics of ATP concentration-dependent uptake of DNP-SG in yeast vacuolar membrane vesicles. The effects of 0 (○), 1 (●), 5 (▽), and 10 mM DTT (▼) on the initial rate of uptake of 10 μM [³H]DNP-SG with increasing concentrations of MgATP were examined in yeast vacuolar membrane vesicles. The data points represent the mean with standard errors of at least three experiments. The data were fit by a least squares linear regression curve that followed the Michaelis–Menten equation ($P < 0.05$) to derive kinetic parameters. The data were further analyzed by a Woolf–Augustinsson–Hofstee plot to illustrate the kinetics of inhibition (inset).

3.4. Demonstration of ATP-dependent GSH transport in rat cLPM vesicles when the DTT concentration is decreased

To examine the effects of DTT on ATP-dependent GSH transport in rat cLPM vesicles, assay conditions had to be established that would keep GSH in the reduced form in the nominal absence of DTT. It is critical to prevent oxidation of GSH to GSSG, as the latter compound is a known substrate for Mrp2 (and Ycf1p). To accomplish this, the transport buffers were degassed under vacuum with vigorous stirring for 30 min at room temperature, and were then bubbled with N$_2$ for an additional 30 min. All subsequent transport reactions were done under

Table 1
Kinetic parameters of ATP-dependent transport of DNP-SG by Ycf1p

[DTT] (mM)	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})
A. Increasing [³H]DNP-SG with 5 mM MgATP		
0	39 ± 2	9.12 ± 0.45
1	28 ± 8	7.00 ± 0.06*
5	40 ± 7	5.70 ± 0.24*
10	44 ± 6	4.80 ± 0.04*
B. 10 μM [³H]DNP-SG with increasing [MgATP]		
0	509 ± 60	0.36 ± 0.02
1	487 ± 140	0.31 ± 0.03
5	444 ± 70	0.24 ± 0.01*
10	855 ± 320	0.13 ± 0.02*

The initial rates of ATP-dependent uptake of increasing concentrations of [³H]DNP-SG with 5 mM MgATP (A), or 10 μM [³H]DNP-SG with increasing concentrations of MgATP (B) were measured in the presence of 0, 1, 5, 10 mM DTT. The kinetic parameters for these transport reactions were derived from data in Figs. 3 and 4. Values represent the mean ± S.E.M. of three experiments. *Significantly different from control, $P < 0.05$.
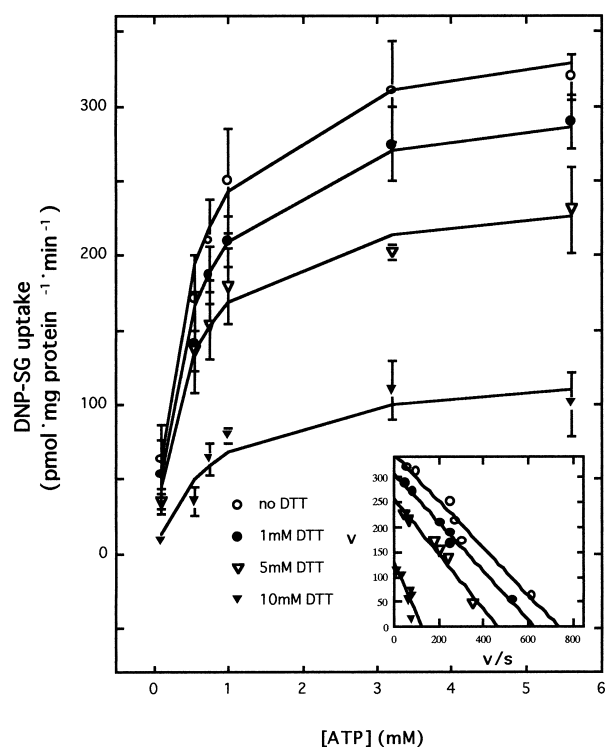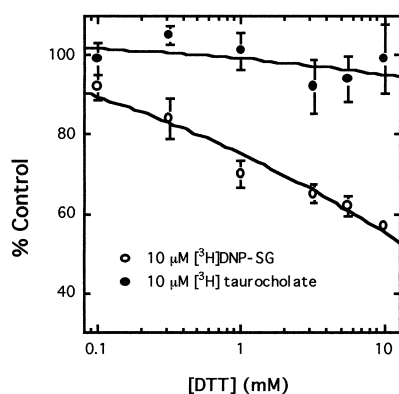
Fig. 5. Effects of DTT on the ATP-dependent uptake of DNP-SG and taurocholate in rat cLPM vesicles. The effects of increasing concentrations of DTT on the initial rate of ATP-dependent uptake of 10 μM [^{3}H]DNP-SG (O) and 10 μM [^{3}H]taurocholate (●) were measured in rat cLPM vesicles. The difference in the uptake after 1 min was expressed as a percentage of control. The control values for the initial rate of ATP-dependent uptake of 10 μM [^{3}H]DNP-SG and 10 μM [^{3}H]taurocholate were 17.5 and 125 pmol (mg protein)$^{-1}$ min^{-1}, respectively.

N_2. The [^{3}H]GSH stock was purchased as a 10 mM DTT solution, and a 100 mM stock of GSH was likewise prepared in 10 mM DTT using N_2-saturated water. The [^{3}H]GSH and unlabeled GSH stocks were diluted with oxygen-free buffers to generate the desired incubation solutions (i.e. 100 μM [^{3}H]GSH with 0.1–10 mM DTT). The GSSG content in either the concentrated GSH stock solutions or the diluted incubation solutions was below the limits of our detec-

tion, < 0.1 μM, for at least 90 min of incubation at 30°C. Moreover, the addition of glutathione reductase with or without NADPH to the yeast vacuolar membrane transport reactions had no effect on ATP-dependent uptake of radioactivity from [^{3}H]GSH (data not shown), also indicating the absence of GSSG in these incubation solutions.

Using these experimental conditions, the uptake of 0.1 mM [^{3}H]GSH by rat cLPM was measured with and without 5 mM ATP, and in the presence of either 10, 1, or 0.1 mM DTT (Fig. 6). In agreement with the inhibitory effect of DTT on DNP-SG transport in cLPM vesicles (Fig. 5), DTT also inhibited ATP-dependent GSH uptake (Fig. 6). The ATP-dependent component of GSH uptake was minimal when measured in the presence of 10 mM DTT (Fig. 6A); however, when the DTT concentration was lowered, the ATP-dependent component of uptake increased, and it became statistically significant at a DTT concentration of 0.1 mM (Fig. 6C). Likewise, the ATP-independent component of GSH uptake increased as DTT concentration was decreased, indicating that this transport process is also sensitive to DTT. However, the molecular nature of this ATP-independent transport process is presently unknown.

4. Discussion

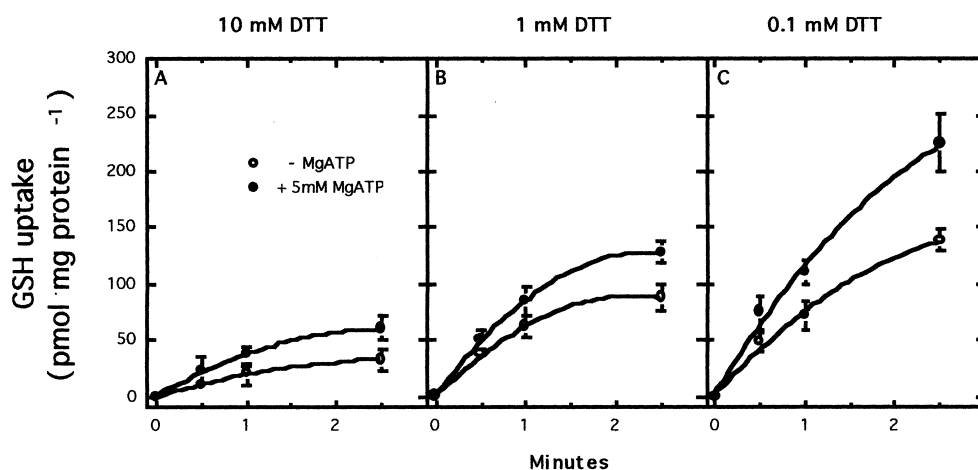The major finding from this study is the direct

Fig. 6. Effects of DTT on the time course of GSH uptake in rat cLPM vesicles. The effect of 10 mM (A), 1 mM (B), or 0.1 mM DTT (C) on the uptake of 0.1 mM [^{3}H]GSH was measured in 50 μg of rat cLPM vesicles in the presence (●) and absence (O) of 5 mM MgATP for up to 2.5 min. The data points represent the mean with standard errors of three experiments.

demonstration of ATP-dependent GSH transport in Mrp2-containing rat liver canalicular membrane vesicles. Detection of ATP-dependent GSH transport activity was made possible only when it was realized that DTT inhibits Mrp2 function. As the concentration of this reducing agent was decreased, the rate of transport increased to a detectable level. Because this reducing agent was used in all previous studies of GSH transport on Mrp proteins, these studies probably underestimated, and in most cases failed to detect a transport signal. DTT also inhibited Ycf1p-mediated GSH and DNP-SG transport (Fig. 2), indicating that our previously reported transport rates in yeast vesicles are also an underestimate [22,23]. However, because the inhibition by DTT is most likely noncompetitive in nature, the published K_m values for these Ycf1p-mediated transport processes remain valid (i.e. a K_m of 0.2 mM for DNP-SG and of 15 mM for GSH).

It is important to point out that the ATP-dependent GSH transport rate measured in rat cLPM vesicles is quite low (Fig. 6), but this is to be expected given the low catalytic efficiency of GSH export. Previous studies suggest that the K_m for GSH transport is high (in the mM range), whereas the V_{max} is only modest [8,15,16,20]. Moreover, cLPM vesicles have a relatively high nonspecific permeability, which leads to the dissipation of solute gradients, as well as a rapid ATP hydrolysis rate, which leads to the loss of the driving force [20,24,27,28]. In addition, because only a small fraction of cLPM vesicles are in the inside-out configuration ($\sim$20% [29]), only a few vesicles contribute to ATP-dependent GSH uptake. A low GSH transport rate was also observed in previous studies with skate liver plasma membrane vesicles [20], with yeast vacuolar membrane vesicles [23], and in cells and membrane vesicles expressing Mrp2 [15–21]. Thus, both vertebrate Mrp2 and yeast Ycf1p are able to transport GSH in an ATP-dependent fashion. This conclusion is consistent with the observation that Mrp2-deficient rats fail to export GSH into hepatic bile, and with other studies in intact cell systems [15–21].

DTT, DTE, and βME inhibited Ycf1p-mediated ATP-dependent transport of DNP-SG in yeast vacuolar membrane vesicles to an equivalent degree, with 10 mM concentrations showing roughly 40% inhibition. DTT produced a quantitatively similar inhibition of Ycf1p- and Mrp2-mediated, ATP-dependent GSH transport. The latter experiments were done under conditions were GSH oxidation to GSSG was minimal (the GSSG concentration was maintained below the detection limit of 0.1 μM). This concentration of GSSG is 3 orders of magnitude lower than the published K_m for GSSG transport on MRP, $\sim$100 μM [33]. In contrast, no effect of DTT was seen on Bat1p-mediated ATP-dependent taurocholate transport in these same yeast membrane vesicles, indicating that DTT's effects are not due to a generalized disruption of transport activity or membrane integrity.

The mechanism of DTT's inhibition of Ycf1p was examined by kinetic analysis of the initial rates of DNP-SG transport in the presence and absence of several concentrations of DTT (Figs. 3 and 4). In the absence of DTT, the apparent K_m for DNP-SG was 39 ± 2 μM and the V_{max} was 9.12 ± 2 nmol (mg protein)$^{-1}$ min^{-1}, and the apparent K_m for ATP was 509 ± 60 μM and the V_{max} was 0.36 ± 0.02 nmol (mg protein)$^{-1}$ min^{-1}. The addition of 1, 5, or 10 mM DTT had no significant effect on the relative affinity (K_m) for either DNP-SG or ATP, but it reduced the maximum velocity (V_{max}), consistent with a noncompetitive model of inhibition. Noncompetitive inhibition by DTT suggests that Ycf1p has at least one thiol-sensitive residue, and that this residue is located at a site distinct from the DNP-SG or ATP binding pockets. Ycf1p contains 18 cysteine residues, but it is presently unknown which of these residues are critical for function.

It had been previously shown that sulfhydryl-reactive reagents such as *N*-ethylmaleimide and 5,5′-dithio-bis(2-nitrobenzoate) are potent inhibitors of ATP-dependent DNP-SG transport in rat liver canalicular plasma membrane vesicles [34,35] and in human erythrocytes [36], suggesting that Mrp2-mediated transport is regulated by critical cysteine residues, and may require thiol/disulfide exchange. The inactivation of DNP-SG transport by 5,5′-dithio-bis(2-nitrobenzoate) was reversed by lengthy treatment with 10 mM DTT, but was only partially reversed when vesicles were pretreated with other thiol-reactive compounds [35]. In contrast, the effects of *N*-ethylmaleimide, which forms a covalent adduct with reduced sulfhydryl groups, were essentially irreversible [35]. The present results demonstrating inhi-

bition by sulfhydryl-reducing agents provides support for the suggestion that Mrp2-mediated transport may be regulated by thiol/disulfide exchange. This model predicts that transport activity should be inhibited by agents that disrupt thiol-redox cycling through either oxidation or reduction reactions. Additional studies are needed to test this hypothesis, and to evaluate the molecular nature and physiological significance of the thiol-sensitive site(s) on Mrp2 and Ycf1p.

Acknowledgements

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