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Preferential Transport of Glutathione *versus* Glutathione Disulfide in Rat Liver Microsomal Vesicles*

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A bi-directional, saturable transport of glutathione (GSH) was found in rat liver microsomal vesicles. GSH transport could be inhibited by the anion transport blockers flufenamic acid and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid. A part of GSH taken up by the vesicles was metabolized to glutathione disulfide (GSSG) in the lumen. Microsomal membrane was virtually nonpermeable toward GSSG; accordingly, GSSG generated in the microsomal lumen could hardly exit. Therefore, GSH transport, contrary to previous assumptions, is preferred in the endoplasmic reticulum, and GSSG entrapped and accumulated in the lumen creates the oxidized state of its redox buffer.

The endoplasmic reticulum (ER)[1] of the cell is the site of the synthesis, posttranslational modification, and folding of proteins transported along the secretory pathway. The oxidizing environment in the lumen of the ER is necessary for the formation of disulfide bonds and for the proper folding of these proteins (1). The oxidative effects are reflected in and supported by the GSH redox buffer; the ratio of GSH and GSSG is around 2:1 within the lumen of ER and along the secretory pathway, whereas the cytosolic ratio ranges from 30:1 to 100:1 (2). However, the primary source(s) of the oxidative effect has not been demonstrated. Recent observations suggest two possible mechanisms. First, the preferential uptake of the oxidized member of a redox couple through the ER membrane and/or the efflux (or exocytosis) of its reduced form could ensure the oxidative environment. Alternatively, enzymes resident in the membrane or lumen of the ER could produce oxidizing compounds (*e.g.* reactive oxygen species) toward the lumen. Experimental evidence supports both mechanisms. Favoring the transport-based hypothesis, the preferential transport of dehydroascorbate (the oxidized form of ascorbate) has been described in rat liver microsomal vesicles (3). Similarly, the selective microsomal transport of GSSG was also reported (2, 4). On the other hand, several microsomal enzymes (cytochrome P-450s, NADPH cytochrome P-450 reductase, gulonolactone oxidase, microsomal iron protein, NADPH-dependent oxidase, sulfydryl oxidase, etc.) can produce reactive oxygen species (5–10). The recent exploration of the ER oxidase protein (ERO1) and its role in the protein folding also support the latter mechanism (11, 12). Because of the conflicting opinions, the microsomal transport of GSH and GSSG has not been unequivocally established. The presently available data are based on the detection of microsome-associated radioactivity by applying a rapid filtration method and radiolabeled compounds (2, 4); however, intraluminal GSH or GSSG contents upon transport have not been directly demonstrated. Therefore, experiments were undertaken to reinvestigate the transport of GSH and GSSG through the ER membrane.

The main difficulties in the investigation of microsomal transport processes are deriving from the very small intraluminal space, the presence of (intraluminal) reactions affecting the transported compounds and in case of several molecules, the significant binding to the membrane. To overcome these problems, glutathione transport was investigated by two different experimental approaches. The light scattering technique (13, 14) allows the real time detection of the permeation of the compounds at high concentrations, whereas with the polyethylene glycol precipitation-rapid sedimentation method (5, 15) high microsomal protein concentrations can be used, which makes the direct detection of the intraluminal pools possible. To distinguish the uptake from the binding to microsomal vesicles the pore-forming compound alamethicin was used (16–18).

EXPERIMENTAL PROCEDURES

Preparation of Rat Liver Microsomes—Rat liver microsomes were prepared from male Sprague-Dawley rats (180–230 g) as described in Ref. 19. Microsomal fractions were resuspended in a buffer containing 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM Mops, pH 7.2. The suspensions were rapidly frozen and maintained under liquid N_2 until required. The latency of mannose 6-phosphatase (20) and *p*-nitrophenol UDP-glucuronosyltransferase (17) activity was greater than 90 and 95%, respectively. Intactness of microsomal membrane was also ascertained by the sustained light scattering signal upon the addition of the poorly permeant sucrose (14). To measure microsomal water space, microsomes were diluted (10 mg protein/ml) in the above buffer containing [^{3}H]H_2O (0.2 μCi/ml) or [^{3}H(C)]inulin (0.17 μCi/ml) and centrifuged, (100,000 × *g* for 60 min), and the radioactivity associated with pellets was measured to enable calculation of extravesicular and intravesicular water spaces (18, 21).

Transport Measurements by Light Scattering Technique—Osmotically induced changes in microsomal vesicle size and shape (13) were monitored at 400 nm at a right angle to the incoming light beam, using a fluorimeter (Perkin-Elmer model 650-10S) equipped with a recorder, a temperature-controlled cuvette holder (22 °C), and a magnetic stirrer, as described elsewhere (14). Briefly, microsomal vesicles (50 μg protein/ml) were equilibrated in a hypotonic medium (5 mM K-Pipes, pH 7.0), and the osmotically induced changes in light scattering were measured

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[1] The abbreviations used are: ER, endoplasmic reticulum; GSH, glutathione; GSSG, glutathione disulfide; Mops, 4-morpholinepropanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; HPLC, high pressure liquid chromatography.

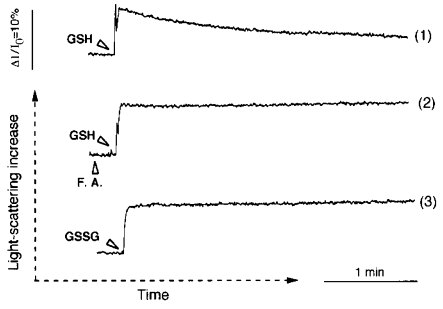

FIG. 1. **Influx of GSH, its inhibition by flufenamic acid, and lack of influx of GSSG in rat liver microsomal vesicles monitored by light scattering.** Light scattering increase is assumed to reflect shrinkage of microsomal vesicles. The addition of poorly permeable solutes results in a sustained shrinkage, and the recovery of the initial signal (swelling phase) after the addition of solutes is assumed to reflect their entry into vesicles (13). Rat liver microsomes (70 μg/ml of protein) were equilibrated in a low osmolarity buffer (5 mM K-Pipes, pH 7) until a stable light scattering base line was obtained. Concentrated solutions of GSH or GSSG (0.5 M, in the K-Pipes buffer, pH 7; *arrows*) were added to 2.0 ml of the microsomal suspensions giving 20 mM final concentration for both compounds. Representative traces out of five to eight similar experiments are shown. *F.A.*, flufenamic acid (2.5 mM).

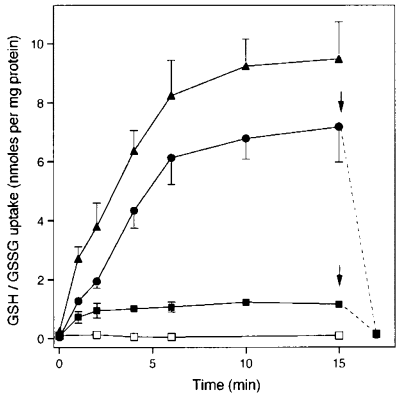

FIG. 2. **Influx of GSH and accumulation of GSSG in rat liver microsomal vesicles.** Microsomes were incubated in the presence of 5 mM GSH (*filled symbols*) or 5 mM GSSG (*open symbols*). At 15 min, alamethicin (0.1 mg/mg microsomal protein) was added to permeabilize the vesicles (*dotted lines*). Intraluminal GSH (*circles*) and GSSG (*squares*) contents were measured as described under "Experimental Procedures." The *triangles* indicate the total uptake expressed as GSH (GSH + 2GSSG) in the presence of 5 mM GSH. Means ± S.E. of four to eight experiments are shown. *Error bars* are not visible when they are smaller than the symbol size.

after the addition of a small volume (<10% of the total incubation volume) of the concentrated and neutralized solutions of the compounds to be tested.

Transport Measurements by Rapid Sedimentation Method—The rapid separation of polyethylene glycol-aggregated microsomes was performed as described previously (5, 15). Briefly, microsomes (10 mg protein/ml) were incubated in the presence of various concentrations of GSH or GSSG in a buffer containing 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM Mops, pH 7.2 at 22 °C. To distinguish the intravesicular and the bound GSH/GSSG, the pore-forming antibiotic alamethicin (16–18) was added at the end of the incubations. At the indicated times 0.5-ml samples were taken and precipitated with 0.1 ml of 25% polyethylene glycol. Microsomal vesicles were rapidly sedimented by centrifugation (20 s at 13000 rpm). Pellets were washed two times with the buffer containing 5% polyethylene glycol. The final pellet was deproteinized with perchloric acid.

Metabolite Measurements—GSH and GSSG contents were measured by HPLC according to Ref. 22. A Waters Alliance HPLC apparatus equipped with autosampler was used; results were analyzed by the Milleneum 2000 software.

Materials—Glutathione, glutathione disulfide, flufenamic acid, alamethicin, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid were obtained from Sigma. [^{3}H]H_2O (1 mCi/g) and [^{3}H(C)]inulin (500 mCi/g) were from NEN Life Science Products. All other chemicals were of analytical grade. Bondapak aminopropyl column (average particle size 10 μm, 300 × 3.9 mm inner diameter) was bought from Waters Millipore (Milford, MA).

RESULTS

First, the permeability of rat liver microsomal membrane toward GSH and GSSG was tested with the light scattering method. Surprisingly, the addition of GSSG resulted in a sustained signal, indicating that microsomal membrane was impermeable toward GSSG, whereas GSH entered the vesicles (Fig. 1). Flufenamic acid, a known anion transport inhibitor (23), hindered GSH permeation in a concentration-dependent way; 2.5 mM flufenamic acid almost completely prevented the influx (Fig. 1).

Thereafter the transport was also detected by measuring the intravesicular GSH and GSSG contents upon their addition by using the polyethylene glycol precipitation-rapid sedimentation method. GSH addition (5 mM, a cytosol-like concentration) resulted in the time-dependent increase of intraluminal GSH content (Fig. 2). An initial rapid phase of uptake was followed by a second slower phase, which in accordance with the light scattering observations did not reach the level of the complete equilibrium estimated on the basis of the measured total intravesicular water space of the microsomes (3.46 ± 0.91 μl/mg protein; mean ± S.D., n = 4). GSH associated with the microsomal vesicles really occupied an intravesicular space, because the addition of the pore-forming alamethicin rapidly released it. GSH taken up by the vesicles was partially oxidized to GSSG. This oxidation process occurred mainly in the first 2 min of the uptake (Fig. 2) and resulted in an approximately 0.3 mM estimated intravesicular concentration of GSSG. The extravesicular GSSG concentration (presumably due to the contamination of added GSH) never exceeded 0.05 mM and did not increase during the incubation. Therefore, at least a 6-fold concentration gradient between the intra- and extravesicular GSSG pools was established, indicating that the oxidation of GSH was predominantly intravesicular. Intravesicularly formed GSSG could also be released from the lumen by alamethicin addition (Fig. 2). Upon the addition of GSSG (5 mM), as expected on the basis of light scattering experiments, a negligible amount of the added compound entered the vesicles (Fig. 2, *open squares*).

The redox potentials of the intravesicular GSH/GSSG redox system were calculated by the Nernst equation from the measured intraluminal GSH and GSSG contents using −0.24 V standard potential for glutathione (2). Concentrations were calculated from the measured contents on the basis of intravesicular water space of rat liver microsomal vesicles (see above). At the second, slower phase of the GSH uptake (after 6 min) the intraluminal redox potential of the GSH/GSSG redox system was stabilized around −0.183 mV, which is close to the value reported in the secretory pathway of an intact cell (2).

The initial rate of GSH uptake was protein concentration-dependent; the addition of ATP in accordance with previous observations (4) did not stimulate GSH transport (data not shown). The influx of GSH was saturable (Fig. 3). No increase in the initial rate (measured after 2 min of incubation) of accumulation of GSH or GSSG was detected above 5 mM extravesicular GSH concentrations (Fig. 3). The apparent Michaelis constant for the total uptake of GSH (GSH + 2GSSG) was 1.65 mM, and the apparent maximal rate was 2.66 nmol/min/mg protein. The correlation coefficient for the linear Lineweaver-Burk plot was r = 0.9904. However, these kinetic data cannot be the exact parameters for the transport alone, because it is also affected by the intravesicular metabolism.

The inhibitory effect of flufenamic acid could be demonstrated in the polyethylene glycol precipitation-rapid sedimen-

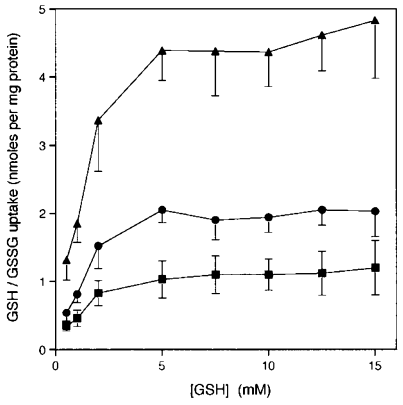

FIG. 3. **Initial rates of the uptake of GSH and accumulation of GSSG in rat liver microsomal vesicles as a function of extravesicular GSH concentration.** The intravesicular content of GSH (*circles*) and GSSG (*squares*) were measured after 2 min of incubation in the presence of the indicated concentration of extravesicular GSH. The *triangles* indicate the total uptake expressed as GSH (GSH + 2GSSG). Means ± S.E. of three experiments are shown.

tation experimental system. 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid, another anion transport inhibitor, also decreased the initial rate of GSH uptake (Table I).

The microsomal GSH transport was bi-directional. After 15 min of incubation in the presence of GSH (5 mM), microsomes were precipitated with polyethylene glycol and were taken up in a GSH-free buffer. GSH release was detected as the decrease of its intravesicular content; the measurements indicated that GSH could leave the lumen of microsomes with a rate comparable with that of influx and flufenamic acid was inhibitory also on the efflux (Fig. 4). GSSG produced by the intraluminal oxidation of GSH could hardly exit from the vesicles (Fig. 4); the half-time of the efflux was eventually longer than 1 h (data not shown).

TABLE I

Inhibition of GSH influx and GSSG accumulation by flufenamic and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acids in rat liver microsomal vesicles

Microsomes were incubated in the presence of 5 mM GSH for 2 min. Intraluminal GSH and GSSG contents were measured as described under "Experimental Procedures." Flufenamic acid and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid were added simultaneously with GSH.

Addition (*n*)	GSH	GSSG
	nmol/2 min/mg protein	
None (6)	2.06 ± 0.30	1.01 ± 0.12
2.5 mM flufenamic acid (3)	0.71 ± 0.09	0.32 ± 0.03
1.0 mM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (3)	0.83 ± 0.07	0.38 ± 0.03

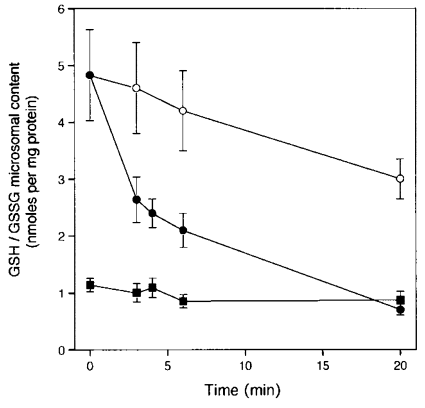

FIG. 4. **Efflux of GSH from rat liver microsomal vesicles and its inhibition by flufenamic acid.** Microsomes were preincubated in the presence of 5 mM GSH for 15 min, and then they were taken up in a GSH-free buffer in the presence or absence of flufenamic acid (2.5 mM). Intraluminal GSH (*circles*) and GSSG (*squares*) contents were measured. *Open circles* indicate efflux experiments performed in the presence of flufenamic acid. Means ± S.E. of four to eight experiments are shown.

DISCUSSION

In this study, we demonstrate that GSH is the preferentially transported form of glutathione in the hepatic endoplasmic reticulum. The features of the transport meet the requirements of a facilitative transport process: it is bi-directional, time-, concentration-, and protein-dependent, saturable, and inhibitable. On the other hand, the very slow permeation of GSSG can be regarded as simple diffusion; the influx and efflux of GSSG are slower than those of sucrose, which is widely used as a nonpermeant test compound for the investigation of the integrity of microsomal membrane. Our results are in contradiction with the conclusion of previous reports indicating the preferential transport of GSSG (2, 4). This discrepancy can be due to the differences in the preparation of microsomes. In one of the studies (2), microsomes were prepared and stored in reducing buffer (*i.e.* in the presence of 1 mM dithiothreitol). The permeability of the microsomal membranes to other low molecular mass compounds was not presented. A possible interpretation might be that an increased nonspecific permeability together with mixed disulfide formation between the reduced microsomal proteins and added GSSG led to the underestimation of GSH and overestimation of GSSG transport. The other study (4) cannot be reconciled with our results due to shortage of data. However, in these reports, the intravesicular GSH and GSSG pools were not directly detected. The major novelties of the present report are (i) the estimation of these intravesicular pools, (ii) the use of alamethicin to distinguish the uptake and binding, and (iii) demonstration of the bi-directional feature of the transport. Our results, gained by two different methods, are consonant. Although the relatively long time required for the precipitation-sedimentation method might allow a partial efflux of the transported compound, the real time detection of the transport with the light scattering technique rules out the possible fast efflux of the investigated compound (especially GSSG) during the washing procedure.

GSH taken up by microsomal vesicles was partially oxidized in the lumen. Because the oxidation was restricted to the early phase of the uptake, it can be attributed to a thiol-disulfide exchange with protein disulfides present in the lumen rather than a continuous oxidation.

As for the transport system(s) involved in the permeability of ER to GSH, it appears to meet the feature of a facilitate transport, *i.e.* there is no energy requirement; it is saturable, bi-directional, and inhibitable. The inhibitors used here, however, do not allow a more precise characterization because they are known to be active on the transport of various anionic compounds (14, 23). Because the transporters of ER have not been completely characterized both functionally and structurally, suggestions cannot be made on the basis of analogies.

In summary, the primary source of the oxidizing environment in the lumen of the endoplasmic reticulum is not the preferential GSSG transport. The intraluminal GSH/GSSG ratio can be generated by the retention of GSSG derived from GSH imported into the ER. GSSG can be formed in the lumen either by GSH-dependent reduction of imported dehydroascorbate (3) catalyzed by protein disulfide isomerase (24) or by other oxidative processes mediated by local enzymes.

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