

# Oxidized Redox State of Glutathione in the Endoplasmic Reticulum

Christopher Hwang,\* Anthony J. Sinskey, Harvey F. Lodish

The redox state of the endoplasmic reticulum (ER) was measured with the peptide *N*-Acetyl-Asn-Tyr-Thr-Cys-NH<sub>2</sub>. The peptide diffused across cellular membranes; some became glycosylated and thus trapped within the secretory pathway, and its cysteine residue underwent reversible thiol-disulfide exchanges with the surrounding redox buffer. Glycosylated peptides from cells were disulfide-linked to glutathione, indicating that glutathione is the major redox buffer in the secretory pathway. The redox state of the secretory pathway was more oxidative than that of the cytosol; the ratio of reduced glutathione to the disulfide form (GSH/GSSG) within the secretory pathway ranged from 1:1 to 3:1, whereas the overall cellular GSH/GSSG ratio ranged from 30:1 to 100:1. Cytosolic glutathione was also transported into the lumen of microsomes in a cell-free system. Although how the ER maintains an oxidative environment is not known, these results suggest that the demonstrated preferential transport of GSSG compared to GSH into the ER lumen may contribute to this redox compartmentation.

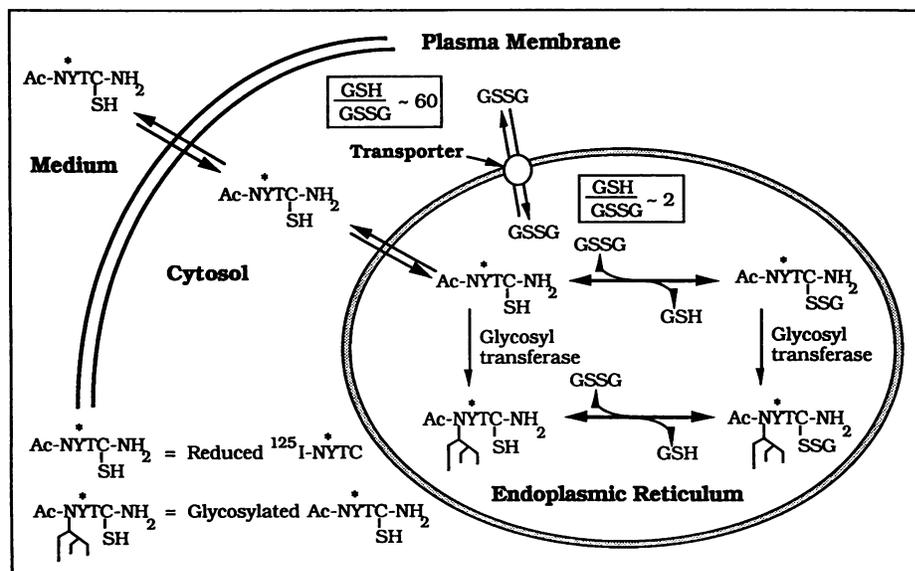
has a consensus sequence (-Asn-X-Thr-) for N-linked glycosylation and should diffuse into the ER and become glycosylated (7, 8). The tyrosine residue can be labeled by iodination and the terminal cysteine residue is expected to undergo SH-SS exchange reactions. Once glycosylated, this peptide remains trapped within the secretory pathway and becomes equilibrated with the redox buffer in these compartments. By identifying the thiol that is linked to such trapped peptides and by analyzing the redox states of them, we found that the principal redox buffer in the secretory pathway indeed is glutathione and that the glutathione redox state of the secretory pathway is more oxidized than that of the cytosol. We also determined that glutathione and cystine were specifically transported into the microsomal lumen from the cytosol. The preferential transport of GSSG relative to GSH is thus one mechanism that generates a more oxidized state in the secretory pathway than in the cytosol.

**Analysis of peptide redox state and glutathione redox state.** To analyze the redox state of glycosylated, endoglycosidase H (Endo H)-sensitive <sup>125</sup>I-labeled NYTC formed in living cells and in microsomes, we developed a simple high-performance thin-layer chromatography (HPTLC) method. Peptides glycosylated in vitro in canine pancreatic microsomes were purified from nonglycosylated peptides on a concanavalin A (Con A) column and then treated with Endo H to remove N-linked sugars (9–12). The redox state of in vitro-glycosylated <sup>125</sup>I-labeled NYTC was analyzed (Fig. 2A). The

Protein refolding studies have shown that disulfide bond formation and rearrangement are reversible thiol-disulfide (SH-SS) exchange reactions and are thermodynamically and kinetically affected by the redox state of the environment. Optimum concentrations of thiol and disulfide are required for rapid and complete refolding of many proteins; the maximum yield of properly folded protein and the rate of renaturation generally occurs at ratios of reduced glutathione to glutathione disulfide (GSH/GSSG) of less than 10 at a physiological concentration of total glutathione (1 to 10 mM) in the presence or absence of protein disulfide isomerase (PDI) (1, 2). These rates were comparable to those observed for protein folding in cultured cells. Moreover, addition of GSSG accelerates folding of proteins translated in vitro in the presence of microsomes, to rates observed in intact cells (3); the amount of GSSG required for rapid folding of these proteins is dependent on the concentration of dithiothreitol (DTT) present in the reaction. This suggests that the redox state—the ratio of thiol to disulfide (SH/SS)—in the reaction affects the folding kinetics of proteins within microsomes. Glutathione is the most abundant nonprotein thiol in mammalian cells and the typical glutathione redox state in cells is too reducing for formation of protein disulfide bonds (4, 5). Thus, the principal redox buffer in the endoplasmic reticulum

(ER) may be glutathione and the ER redox state may be more oxidized than that of the cytosol (2, 6). However, there has been no demonstration of the presence of glutathione in the ER, nor measurement of the redox state within that organelle.

We have developed accurate methodologies for the measurement of SH/SS redox states of cultured cells and of their secretory pathway. To measure the latter, we used the tetrapeptide *N*-Acetyl-Asn-Tyr-Thr-Cys-NH<sub>2</sub> (NYTC) (Fig. 1). This peptide



**Fig. 1.** Schematic diagram for glycosylation of NYTC by cultured cells. Cells were incubated with <sup>125</sup>I-labeled NYTC. The cells were then lysed and the cellular redox state was simultaneously quenched (15). Glycosylated NYTC was purified from cells and deglycosylated; its redox state was analyzed by HPTLC. On the basis of an established redox standard curve, the secretory pathway redox state was extrapolated from the NYTC redox state. To identify the principal secretory pathway redox buffer, the thiol linked to glycosylated NYTC was identified.

The authors are in the Department of Biology, Whitehead Institute for Biomedical Research, and Biotechnology Process Engineering Center, Massachusetts Institute of Technology, Cambridge, MA 02142.

\*Present address: Genzyme Corp., One Mountain Road, Framingham, MA 01701-9322.

$^{125}\text{I}$ -labeled NYTC disulfide bonded to glutathione (NYTC-SSG) or to cysteine (NYTC-CyS) was easily separated from  $^{125}\text{I}$ -labeled NYTC that was reduced and derivatized with *N*-ethylmaleimide (NEM) or from  $^{125}\text{I}$ -labeled NYTC disulfide bonded to cysteamine (NYTC-CyM). The equilibrium constant,  $K_{\text{eq}}$ , for the SH-SS exchange reaction between glycosylated NYTC and a glutathione redox buffer (Eqs. 1 and 2) was determined. NYTC glycosylated in microsomes was incubated in solutions containing various ratios of GSH to GSSG.

GSSG + NYTC-SH



$$K_{\text{eq}} = \frac{[\text{GSH}][\text{NYTC-SSG}]}{[\text{GSSG}][\text{NYTC-SH}]} \quad (2)$$

The peptide redox state was analyzed by HPTLC, and the GSH/GSSG ratio was measured enzymatically (13). The relations between the two redox systems is fairly linear and for the range of GSH/GSSG ratios in the ER lumen (<10; see below), the slope, which corresponds to the  $K_{\text{eq}}$  between the two redox systems, is 1.82 with a correlation coefficient of 0.99 (Fig. 2B). This value of  $K_{\text{eq}}$  was expected, since NYTC is a small and relatively uncharged peptide, and the function of its sulfhydryl group should be similar to that of cysteine.

NYTC was also glycosylated in cultured CRL-1606 hybridoma cells (Table 1). When cells were incubated in medium containing reduced  $^{125}\text{I}$ -labeled NYTC (1.5  $\mu\text{M}$ ) at 37°C for 2 hours, approximately 1.5 percent of added NYTC was recovered from cells, of which approximately 5 percent bound to a column of Con A. Similar peptides, without the cysteine residue, dif-

fuse across membranes and become glycosylated in chicken oviduct microsomes (7) and in Chinese hamster ovary (CHO) and HepG2 cells (8). In cells treated with cycloheximide, an inhibitor of protein synthesis, six times more glycosylated  $^{125}\text{I}$ -labeled NYTC was formed. Presumably, when protein synthesis was blocked, fewer proteins were available in the ER to compete for glycosylation of  $^{125}\text{I}$ -labeled NYTC. Much less glycosylated  $^{125}\text{I}$ -labeled NYTC was formed after treatment of cells with tunicamycin, an inhibitor of N-linked glycosylation; incubation was incomplete presumably because the 1-hour preliminary incubation was insufficient to deplete all precursors of N-linked oligosaccharides. Finally, addition of excess unlabeled peptide to cells inhibited glycosylation of labeled peptides, evidence that the N-linked glycosylation machinery in the ER is saturable.

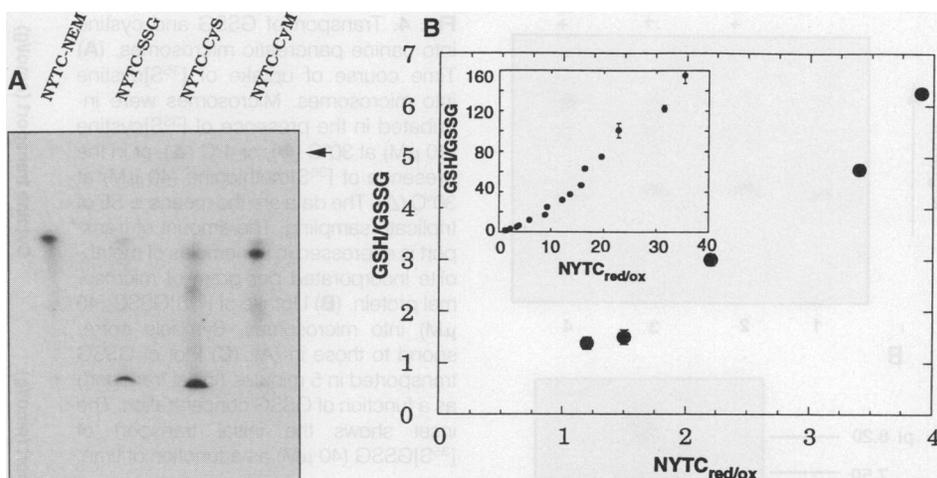
Glycosylated NYTC cannot diffuse across membranes because of the hydrophilic nature of the oligosaccharides (8, 14). However, some might traverse the secretory pathway and be secreted. Indeed, analysis

of  $^{125}\text{I}$ -labeled peptides in the culture medium indicated that approximately 40 percent of the Endo H-sensitive (that is, glycosylated) peptide was secreted by cells. This suggests that measurements obtained from the redox state of glycosylated Endo H-sensitive  $^{125}\text{I}$ -labeled NYTC recovered from cells were those of the SH-SS redox state in the secretory pathway as a whole, and not specifically that in the ER.

**Identification of the principal redox buffer in the ER.** We propose that the molecule disulfide-linked to glycosylated  $^{125}\text{I}$ -labeled NYTC in cultured cells is the principal redox buffer of the secretory pathway. Glycosylated  $^{125}\text{I}$ -labeled NYTC recovered from cells (15) was treated with Endo H and analyzed to identify the NYTC-mixed disulfide; the mixed disulfide was identified by a combination of HPTLC and isoelectric focusing (IEF) gel electrophoresis (16) separation techniques. Although HPTLC did not separate standard  $^{125}\text{I}$ -labeled NYTC-SSG (Fig. 3A, lane 2) from NYTC-CyS (lane 3), the Endo H-digested glycosylated peptide recovered from

**Table 1.** Glycosylation of  $^{125}\text{I}$ -labeled NYTC by hybridoma CRL-1606. Murine hybridoma CRL-1606, resuspended in fresh medium ( $1 \times 10^7$  to  $2 \times 10^7$  cells/ml), were treated, during constant agitation, with cycloheximide (100  $\mu\text{g/ml}$ ), tunicamycin (10  $\mu\text{g/ml}$ ), or excess unlabeled NYTC (50 times the concentration of  $^{125}\text{I}$ -labeled NYTC) for 1 hour at 37°C. Reduced  $^{125}\text{I}$ -labeled NYTC (1.5  $\mu\text{M}$ , total of  $3 \times 10^7$  cpm) was added and the incubation continued for 2 hours. Cells were then sedimented and lysed in 0.5% Triton X-100, sonicated, and treated with 20 mM DTT at 50°C for 1 hour. Subsequently, the glycosylated NYTC in cells was quantified by Con A-Sepharose chromatography.

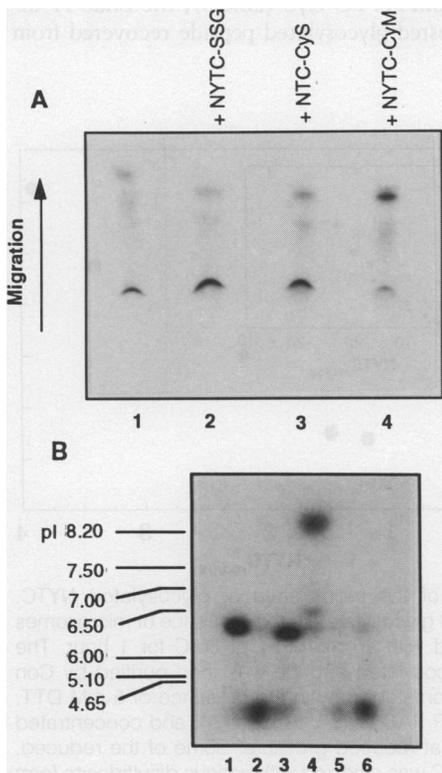
Conditions	$^{125}\text{I}$ -labeled NYTC	
	Total cells ( $10^5$ cpm)	Bound to Con A (percent)
Control	4.5	5.1
Cycloheximide	7.0	18.3
Tunicamycin	3.9	2.4
Unlabeled NYTC	4.3	1.0



**Fig. 2.** Analysis of the redox state of glycosylated NYTC.  $^{125}\text{I}$ -labeled NYTC glycosylated in the presence of microsomes (11) was reduced with 20 mM DTT at 50°C for 1 hour. The reduced and glycosylated peptide was then purified by Con A-Sepharose chromatography in the presence of 5 mM DTT, desalted on a SEP PAC C-18 cartridge (9), and concentrated by centrifugation at reduced pressure. Some of the reduced, glycosylated NYTC was oxidized with various disulfides to form disulfide-linked glycosylated NYTC (35). Derivatives of both the reduced and disulfide-linked glycosylated  $^{125}\text{I}$ -labeled NYTC peptides were obtained by treatment with NEM (Sigma), (pH 8.5) (1000-fold molar excess); the derivatives were purified on SEP PAC C-18 cartridges, concentrated, and digested with Endo H overnight. The peptides were then repurified and analyzed on cellulose HPTLC plates (EM Science). The solvent system contained 69 percent acetonitrile and 0.1 percent TFA in water. After the plates were dried, they were exposed to Kodak XAR-5 film and developed (A). Arrow at top indicates the solvent front, and arrow at bottom indicates the origin. Migration was from bottom to top. (B) Relation between NYTC and glutathione redox states. The reduced, glycosylated  $^{125}\text{I}$ -labeled NYTC (<0.2 nM) was incubated in 10 mM total glutathione at different GSH/GSSG ratios for 30 minutes at 30°C. The samples were then derivatized (36). The peptide samples were prepared and analyzed as described in (A). The autoradiographs were scanned (Molecular Dynamics Laser Scanner; Molecular Dynamics) coupled to Image-quant™ software. Triplicate samples were obtained for analysis of the ratio of GSH to GSSG, whereas duplicate samples were obtained for peptide redox state analysis. The inset shows the same standard curve with expanded redox range.

cells (lane 1) was distinguished from NYTC-CyM (lane 4). Further separation was provided by IEF (Fig. 3B), in which NYTC-SSG (lane 2) was separated from NYTC-CyS (lane 3) and NYTC-CyM (lane 4). This separation occurs because glutathione contains a glutamate residue, which gives the NYTC-SSG a more acidic isoelectric point (PI) than NYTC-CyS. The disulfide-linked glycosylated NYTC in CRL-1606 cells (lane 5) comigrated with NYTC-SSG (lane 6), an indication that the principal redox buffer in the secretory pathway and in the ER is glutathione. In that all disulfide-linked glycosylated  $^{125}\text{I}$ -labeled NYTC recovered from the hybridoma cells were disulfide bonded to glutathione, this result is consistent with our finding that glutathione accounts for more than 99 percent of all nonprotein thiols in these cells (17).

Two identical cell cultures were used to measure the total cellular and secretory



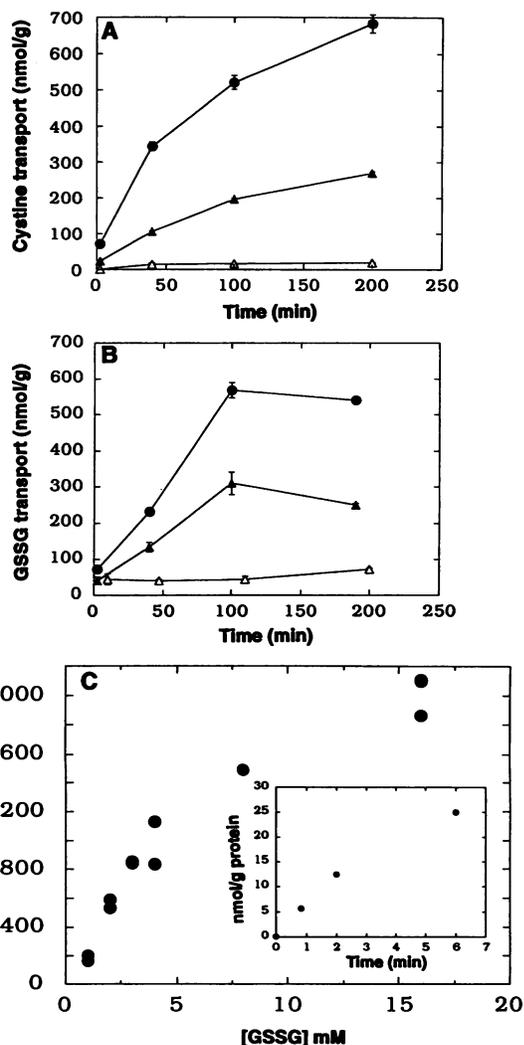
**Fig. 3.** Identification of NYTC mixed disulfide by high-performance thin-layer chromatography and isoelectric focusing. Glycosylated  $^{125}\text{I}$ -labeled NYTC purified from CRL-1606 cells (15) and treated with Endo H (cell sample) was analyzed by HPTLC (A) and IEF (B) along with  $^{125}\text{I}$ -labeled NYTC mixed disulfide standards (35). (A) Lane 1, cell sample only; lane 2, cell sample with NYTC-SSG standard; lane 3, cell sample with NYTC-CyS standard; lane 4, cell sample with NYTC-CyM standard. (B) Lane 1, NYTC-NEM standard; lane 2, NYTC-SSG standard; lane 3, NYTC-CyS standard; lane 4, NYTC-CyM standard; lane 5, cell sample; lane 6, cell sample with NYTC-SSG standard.

pathway redox states. One culture, incubated with  $^{125}\text{I}$ -labeled NYTC, was used to measure the secretory pathway redox state (15). The second culture, without  $^{125}\text{I}$ -labeled NYTC, was used to determine intracellular GSH/GSSG ratios (18) (Table 2). The measured intracellular GSH/GSSG ratios ranged from 30:1 to 100:1, which corresponds to a redox potential of approximately  $-221$  to  $-236$  mV (19). In contrast, the ratio of GSH to GSSG in the secretory pathway, as determined from the redox state of Endo H-sensitive, glycosylated  $^{125}\text{I}$ -labeled NYTC (Fig. 3A) and the  $K_{\text{eq}}$  (Fig. 2B) ranged from approximately 1:1 to 3:1. This corresponds to redox potentials of approximately  $-170$  to  $-185$  mV, if one assumes the total glutathione concentration in the secretory pathway is 8 mM, or approximately  $-133$  to  $-165$  mV if the concentration is 1 mM.

The GSH-GSSG redox state of the secretory pathway is more oxidized than that of the cytosol, and is similar to the redox environment known to be optimum for refolding of disulfide-bonded proteins. Specifically, the GSH/GSSG ratios for the secre-

tory pathway are in agreement with the redox environment optimum for PDI catalysis of oxidative folding of ribonuclease (RNase)—1 mM GSH and 0.2 mM GSSG (2). Changing the ratio of GSH/GSSG affect the availability of the proper substrate for PDI and also the redox state of PDI; both conditions affect the rate of folding of disulfide-bonded proteins (2). By extrapolation of the results of (2), the optimal redox potential for PDI-catalyzed RNase refolding is approximately  $-165 \pm 5$  mV; assuming a glutathione concentration of 1 to 10 mM in the ER, a GSH/GSSG ratio of 2:1 in the secretory pathway would result in 80 to 100% of the maximum rate of PDI-catalyzed RNase folding. If the GSH/GSSG ratio and glutathione concentration in the ER were similar to those in the cytosol, little (<5 percent) or no refolding of RNase in the presence or absence of PDI would be expected. These analyses explain why *Escherichia coli* cannot properly fold recombinant proteins with disulfide bonds; typical GSH/GSSG ratios for *E. coli* are in the range of 50:1 to 200:1 (20), which is in agreement with the estimated redox potential of

**Fig. 4.** Transport of GSSG and cystine into canine pancreatic microsomes. (A) Time course of uptake of  $^{35}\text{S}$ cystine into microsomes. Microsomes were incubated in the presence of  $^{35}\text{S}$ cystine ( $40 \mu\text{M}$ ) at  $30^\circ\text{C}$  ( $\bullet$ ), or  $4^\circ\text{C}$  ( $\blacktriangle$ ), or in the presence of  $^{35}\text{S}$ methionine ( $40 \mu\text{M}$ ) at  $30^\circ\text{C}$  ( $\Delta$ ). The data are the means  $\pm$  SE of triplicate sampling. The amount of transport is expressed in nanomoles of metabolite incorporated per gram of microsomal protein. (B) Uptake of  $^{35}\text{S}$ GSSG ( $40 \mu\text{M}$ ) into microsomes. Symbols correspond to those in (A). (C) Plot of GSSG transported in 5 minutes (initial transport) as a function of GSSG concentration. The inset shows the initial transport of  $^{35}\text{S}$ GSSG ( $40 \mu\text{M}$ ) as a function of time.



**Table 2.** Comparison of cellular redox state with secretory pathway redox state in hybridoma CRL-1606 (15).

Cultures	GSH/GSSG			
	Redox		Secretory pathway†	
	Cellular (ratio)	mV*	Ratio	mV*
1	61 ± 7	(-233)	2.8 ± 0.5	(-185)
2	57 ± 7	(-232)	2.3 ± 0.2	(-181)
3	97 ± 15	(-239)	3.3 ± 1.2	(-188)
4	31 ± 1	(-223)	1.5 ± 0.8	(-172)
5	66 ± 3	(-234)	1.8 ± 0.4	(-176)

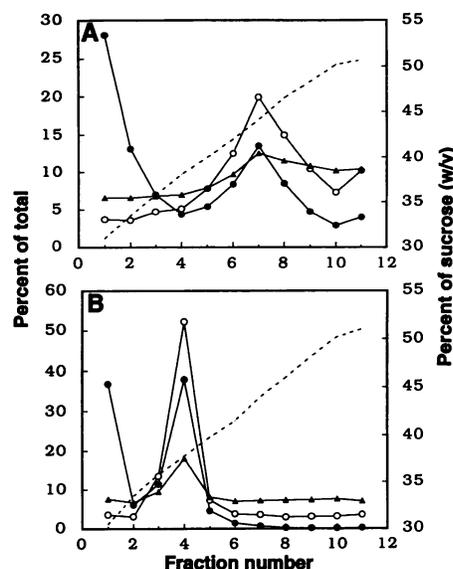
\*Redox potential calculated from the Nernst equation with a total glutathione concentration of 8 mM and a standard redox potential of  $-0.24$  V (19). †These ratios were calculated from the measured redox state of the glycosylated and Endo H-sensitive  $^{125}$ I-labeled NYTC purified from cells with the  $K_{eq}$  of 1.82 (Fig. 2B).

$\sim -0.26$  to  $-0.28$  V calculated from the redox state of *E. coli* thioredoxin (4). Our findings are also in concordance with the redox properties of cytosolic thioredoxin and PDI in the ER, which have standard redox potentials of  $-0.26$  V (21) and  $-0.11$  V (6), respectively. The former is similar to the redox potential of the cytosol in CRL-1606 cells and the latter to that of the secretory pathway.

**Microsomal uptake of glutathione, cysteine, and cystine.** Because enzymes catalyzing synthesis of glutathione are found only in the cytosol (22), there must exist a transport mechanism for transporting cytosolic glutathione into the ER lumen. Because glutathione is a charged molecule, it is unlikely to diffuse through the phospholipid bilayer from the cytosol into the ER lumen (23). The fact that addition of (cytosolic) glutathione disulfide (GSSG) accelerates folding of in vitro-translated proteins within the lumen of microsomes suggests that GSSG is transported into the ER lumen (3). Furthermore, addition of GSSG to rat liver microsomes stimulates glutathione S-transferase activity in the microsomes (24). GSH and GSSG are also transported into mitochondria (25).

Both  $^{35}$ S-labeled cystine and GSSG (40

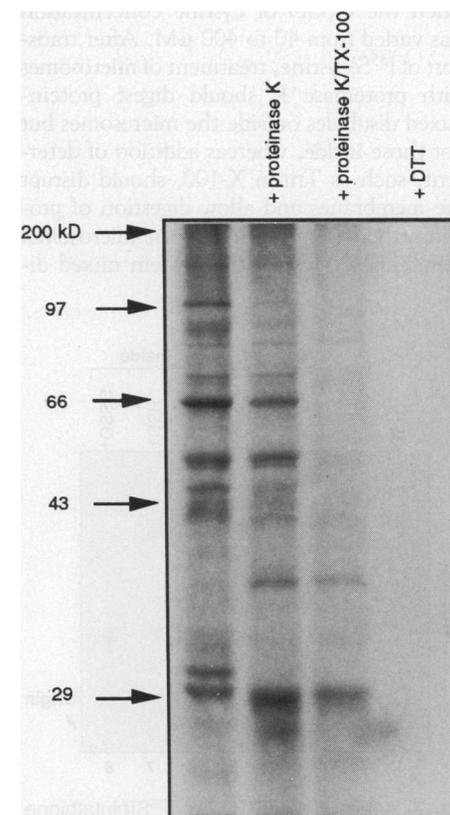
$\mu$ M) were taken up into intact canine pancreatic microsomes in a time-dependent manner (Fig. 4, A and B) (26, 27). Transport was temperature-sensitive and appeared to be specific because methionine was not transported into microsomes. Approximately 60 to 90 percent of the radioactivity taken up by the microsomes was released when the microsomes were treated with Triton X-100 (0.1 percent) in phosphate-buffered saline (PBS). Microsomal uptake of  $^{35}$ S-labeled GSSG and cystine into microsomes was saturable (Fig. 4C). The apparent Michaelis constants ( $K_m$ ) for



**Fig. 5.** Sucrose density fractionation of microsome reactions after transport of  $^{35}$ S]cystine. Microsomes were incubated in the presence of  $^{35}$ S]cystine ( $40 \mu$ M) for 90 minutes, treated with 1 mM NEM on ice for 10 minutes, and treated with (B) or without (A) RNase (37). Microsomes were then centrifuged through a cushion of 0.7 M sucrose, resuspended gently in transport buffer, and layered on a sucrose gradient. After centrifugation, the gradients were fractionated, and the radioactivity ( $\bullet$ ),  $\alpha$ -glucosidase activity ( $\circ$ ), protein concentration ( $\blacktriangle$ ), and sucrose concentration (-----) were quantitated for each fraction.

GSSG and cystine transport were 9 and 1 mM, respectively; the apparent maximal rates ( $V_{max}$ ) were 3000 and 800 nm per gram of protein per minute, respectively. The correlation coefficients for the linear Lineweaver-Burk plots for GSSG and cystine transport were 0.96 and 0.94, respectively. These may not be kinetic parameters for transport alone because the initial transport analysis is complicated by formation of mixed disulfides in the lumen. The rates of transport of GSH and cysteine are approximately ten times slower than those of their corresponding disulfides (Table 3).

Because our microsome preparation could contain non-ER vesicles, it was necessary to demonstrate that the transported GSSG and cystine indeed were associated with ER membranes. The  $^{35}$ S]cystine appeared to be incorporated into rough ER fractions because  $\alpha$ -glucosidase, an oligosaccharide-cleaving enzyme localized to the



**Fig. 6.** Nonreducing SDS-PAGE analysis of protein mixed disulfides formed upon transport of  $^{35}$ S]cystine into microsomes. Microsomes were incubated in the presence of  $^{35}$ S]cystine ( $40 \mu$ M) at  $30^\circ$ C for 2 hours. Transport was then terminated by the addition of 1 mM NEM; then the microsomes were subjected to no treatment (lane 1), proteinase K digestion (lane 2) (38), proteinase K digestion in the presence of 1 percent Triton X-100 (lane 3), or reduction by 20 mM DTT at  $95^\circ$ C for 5 minutes (lane 4). The samples were then analyzed on non-reducing SDS-PAGE (39).

**Table 3.** Comparison of microsomal uptake of GSSG and cystine with their corresponding thiols. The rates of initial microsomal uptake of  $^{35}$ S]GSH,  $^{35}$ S]GSSG,  $^{35}$ S]cystine, and  $^{35}$ S]cystine were determined at  $30^\circ$ C for 5 minutes. The rates were expressed as nanomoles per gram of microsomal protein per minute. The redox state of added  $^{35}$ S-labeled thiols or disulfides were analyzed after transport, and no significant change in their redox states was observed (34).

Solution	Uptake (nmol $g^{-1}$ $min^{-1}$ )
1 mM GSSG	300 ± 19
1 mM GSH	32 ± 15
1 mM cystine	332 ± 17
1 mM cysteine	32 ± 10

rough ER, cofractionated with the incorporated [<sup>35</sup>S]cystine, whether or not microsomes were treated with RNase (Fig. 5). Treatment with RNase stripped ribosomes from the ER membranes and resulted in a decrease in the density of the membranes, but did not affect the density of other subcellular fractions such as the Golgi or lysosomes. Similar results were obtained for equilibrium density-gradient centrifugation of microsomes treated with <sup>35</sup>S-labeled GSSG (20).

Nonreducing SDS-polyacrylamide gel electrophoresis (PAGE) analysis of microsomes incubated with [<sup>35</sup>S]cystine (40 μM) for 2 hours at 30°C revealed that transported [<sup>35</sup>S]cystine formed mixed disulfides with microsomal proteins (Fig. 6). Once transported into microsomes, most of the GSSG and cystine formed mixed disulfides with microsomal proteins; approximately 70 to 80 percent of <sup>35</sup>S-labeled GSSG and [<sup>35</sup>S]cystine were precipitable with sulfosalicylic acid (SSA) (10 percent w/v) (28). This percentage did not change when the GSSG or cystine concentration was varied from 40 to 400 μM. After transport of [<sup>35</sup>S]cystine, treatment of microsomes with proteinase K should digest protein-mixed disulfides outside the microsomes but not those inside, whereas addition of detergent, such as Triton X-100, should disrupt the membranes and allow digestion of proteins otherwise protected by the microsomal membranes. Most of the protein mixed di-

sulfides were protected from protease digestion (Fig. 6) but were subsequently digested after addition of Triton X-100. Thus, most of these mixed disulfides were on the luminal face of the microsomes. These protein mixed disulfides appeared to be reducible because treatment of samples with DTT resulted in disappearance of labeled protein bands on SDS-PAGE (Fig. 6). Similar results were obtained for the transport of <sup>35</sup>S-labeled GSSG (20). As a control, in vitro-translated prolactin was completely digested by proteinase K, whereas microsome-processed prolactin was resistant (20). Incorporation of <sup>35</sup>S-labeled GSSG or cystine into proteins through biosynthesis was ruled out by the presence of cycloheximide in the transport buffer; a similar experiment with [<sup>35</sup>S]methionine did not result in any labeled protein bands after SDS-PAGE (20).

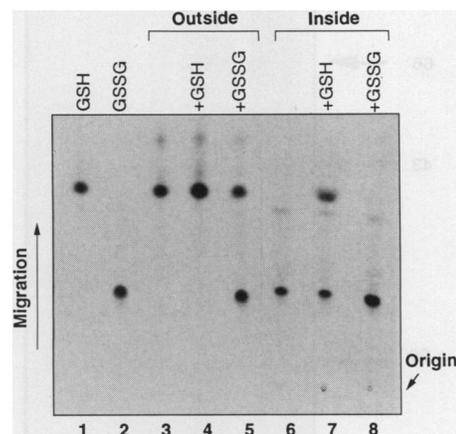
**Comparison of intramicrosomal and extramicrosomal redox states.** To demonstrate that the redox potential within the

microsomes was more oxidized than that outside (the cytosol), microsomes were incubated in 4 mM reduced <sup>35</sup>S-labeled glutathione, ~0.4 mM oxidized <sup>35</sup>S-labeled glutathione, and 1 mM DTT to generate a GSH/GSSG ratio outside the microsomes similar to that normally found in the cytosol of mammalian cells. After incubation at 30°C for 2 hours, virtually all of the <sup>35</sup>S-labeled glutathione outside of the microsomes was in the reduced form whereas most of the <sup>35</sup>S-labeled glutathione inside the microsomes was in the oxidized form and comigrated with a <sup>35</sup>S-labeled GSSG standard (Fig. 7). The resultant GSH/GSSG ratios determined from this experiment indicated that the GSH/GSSG ratio inside the microsomes was two to three orders of magnitude lower than that outside (Table 4.)

Comparison of intramicrosomal and extramicrosomal redox states were also obtained with the peptide redox probe NYTC. Nonradioactive glutathione was used and the microsomes were incubated in the presence of <sup>125</sup>I-labeled NYTC (10 nM). The GSH/GSSG ratios determined from the redox state of the glycosylated NYTC from inside the microsomes were compared to those determined from the unglycosylated NYTC outside the microsomes (Table 4). Typical NYTC-SH/NYTC-SSG and GSH/GSSG ratios inside the microsomes were one to two orders of magnitude lower than those outside. These results are in qualitative agreement with the <sup>35</sup>S-labeled glutathione uptake study (Table 4) and with the cellular and secretory pathway GSH/GSSG ratios in CRL-1606 cells (Table 2). Similar results were obtained for incubation of microsomes in a 0.4 mM cystine-cysteine redox buffer (20).

We conclude that the principal redox buffer in the ER is glutathione and that its redox state is approximately 20 to 100 times more oxidized than that of the cytosol. These conclusions are in agreement with in vitro protein refolding studies and can be justified because disulfide bond formation is a net oxidation reaction and thus would be thermodynamically and kinetically unfavorable under the reducing environment normally found in the cytosol. Although the glutathione concentration in the ER could not be measured, GSSG transport studies with microsomes suggest that GSSG is equilibrated between the ER and the cytosol by a transporter. Thus, we estimate the GSH concentration in the ER to be approximately 0.5 to 1 mM, a value in agreement with studies on PDI catalysis of RNase refolding, which exhibits a *K<sub>m</sub>* of 0.4 to 0.6 mM for GSH (29).

The compartmentation of the glutathione redox state in mammalian cells suggests the importance of the glutathione



**Fig. 7.** Analysis by HPTLC of [<sup>35</sup>S]glutathione transported inside and remaining outside of microsomes. Microsomes were incubated in the presence of 4 mM [<sup>35</sup>S]glutathione, 4 mM ATP, and 1 mM DTT at 30°C. After 2 hours, samples from inside and outside the microsomes were prepared and analyzed (40). Lane 1, [<sup>35</sup>S]GSH standard; lane 2, [<sup>35</sup>S]GSSG standard; lane 3, sample from outside the microsomes (sample<sub>out</sub>); lanes 4 and 5, sample<sub>out</sub> with [<sup>35</sup>S]GSH or [<sup>35</sup>S]GSSG standards, respectively; lane 6, sample from inside the microsomes of the same experiment (sample<sub>in</sub>); lanes 7 and 8, sample<sub>in</sub> with [<sup>35</sup>S]GSH or [<sup>35</sup>S]GSSG standards, respectively.

**Table 4.** Comparison of intramicrosomal and extramicrosomal GSH/GSSG redox state. Data for the glutathione redox states of the cytosol and the lumen, established after uptake of <sup>35</sup>S-labeled glutathione, at different GSH/GSSG ratios, are presented as means ± SE for duplicates. For the data presented for the uptake of NYTC, the incubation conditions were as described for uptake of labeled GSSG except nonradioactive GSH was used, 10 nM of reduced <sup>125</sup>I-labeled NYTC was added, and a lower microsome concentration (<1 mg/ml) was used. The peptide samples were prepared as in the whole-cell system (15) except nonglycosylated NYTC from outside the microsomes was used for the determination of the cytosolic redox state.

Uptake of <sup>35</sup> S-labeled glutathione (GSH/GSSG)			
Cytosol		Lumen	
121 ± 34		1.7 ± 0.9	
164 ± 5		0.6 ± 0.3	
47 ± 0		0.7 ± 0.3	
Uptake of <sup>125</sup> I-labeled NYTC			
Cytosol		Lumen	
NYTC-SH/SSG* (measured)	GSH/GSSG† (calculated)	NYTC-SH/SSG‡ (measured)	GSH/GSSG† (calculated)
52	224	7.9	15
40	168	2.2	3.1
21	86	2.1	3.0
18	69	4.7	8.5

\*The ratio of reduced to glutathione-linked, unglycosylated <sup>125</sup>I-labeled NYTC outside the microsomes. †GSH/GSSG ratios were extrapolated from the measured <sup>125</sup>I-labeled NYTC redox state and from the redox state standard curve in Fig. 2B. ‡The ratio of reduced to glutathione-linked, glycosylated <sup>125</sup>I-labeled NYTC from inside the microsomes.

redox states (4, 5, 30). Although glutathione reductase and the availability of GSSG and NADPH determine the high GSH/GSSG ratios in the cytosol (4), how the ER is maintained in a much more oxidative state is not known. Preferential transport of GSSG over GSH from the cytosol into the ER lumen may contribute to the establishment of the oxidative environment in the ER, and the cytosol appears to be the source of glutathione in the ER. Other mechanisms that have been demonstrated to form oxidizing equivalents in the ER include a NADPH-dependent oxidase system (31), the vitamin K redox cycle (32), and the sulfhydryl oxidase system (33).

## REFERENCES AND NOTES

- J. G. L. Petersen and K. J. Dorrington, *J. Biol. Chem.* **249**, 5633 (1974); V. P. Saxena *et al.*, *Biochemistry* **9**, 5015 (1970); D. B. Wetlauffer *et al.*, *Protein Eng.* **1**, 141 (1987); J. Koivu and R. Myllyla, *J. Biol. Chem.* **262**, 6159 (1987).
- M. M. Lyles and H. F. Gilbert, *Biochemistry* **30**, 613 (1991).
- N. J. Balleid and R. B. Freedman, *Nature* **335**, 649 (1988); M. A. Kaderbhai and B. M. Austen, *Eur. J. Biochem.* **153**, 167 (1985); G. Scheele and R. Jacoby, *J. Biol. Chem.* **257**, 12277 (1982); G. Scheele and R. Jacoby, *ibid.* **258**, 2005 (1983).
- H. F. Gilbert, *Adv. Enzymol.* **63**, 69 (1990).
- N. S. Kosower and E. M. Kosower, *Int. Rev. Cytol.* **54**, 109 (1978).
- H. C. Hawkins, M. D. Nardi, R. B. Freedman, *Biochem. J.* **275**, 341 (1991).
- J. K. Welply *et al.*, *J. Biol. Chem.* **258**, 11856 (1983).
- F. T. Weiland *et al.*, *Cell* **50**, 289 (1987).
- The peptide NYTC was synthesized and purified as described [E. K. O'Shea *et al.*, *Science* **243**, 538 (1989)] except that it was reduced with 0.5 M DTT at pH 8.0 prior to desalting on a Sephadex G10 column. The cysteine residue of the peptide was protected from oxidation to the aldehydic amino acid during iodination by the formation of a mixed disulfide with GSSG (Boehringer Mannheim). Initially, NYTC was bound to SEP PAC C-18 cartridge (Waters), and excess DTT was removed by washing with 0.1 percent trifluoroacetic acid (TFA) in water. The peptide was then eluted with a solution of acetonitrile (90 percent), H<sub>2</sub>O (10 percent), and TFA (0.1 percent). It was lyophilized and then resuspended in excess (80 times the peptide molar concentration) GSSG in 100 mM tris (pH 8.5), incubated at 40°C until thiols were no longer detectable by a sulfhydryl assay [S. Bannai and T. Ishii, *J. Cell. Physiol.* **104**, 215 (1980)], repurified on a SEP PAC C-18 cartridge, dissolved in 10 mM Hepes (pH 7.4), and iodinated (IODOGEN; Pierce Chemical). The iodinated peptide was purified on a SEP PAC C-18 cartridge, centrifuged at reduced pressure, and reduced with 50 mM DTT in 100 mM tris (pH 8.5). The iodinated peptide was then separated from the uniodinated peptide by HPLC with a linear acetonitrile-H<sub>2</sub>O gradient containing 0.1 percent TFA, stored in 0.1 percent TFA in water at -75°C, and used within 3 weeks. The specific radioactivity of the iodinated peptide was approximately 2000 to 4000 Ci/mmmole.
- Canine pancreatic microsomes were prepared as described [M. A. Kaderbhai and B. M. Austen, *Biochem. J.* **217**, 145 (1984)] except that Trasylol was not included in the homogenization buffer and the steps with a tissue press and a Sepharose CL-2B column were omitted. The microsomes were treated with nuclease [P. Walter and G. Blobel, *Methods Enzymol.* **96**, 84 (1983)] and stored in 0.25 M sucrose in 20 mM Hepes (pH 7.4) and 1 mM DTT. The stored microsomes were stable for at least 6 months in liquid nitrogen. The protein concentration of the prepared microsomes was approximately 8 to 12 mg/ml.
- Iodinated NYTC (<10 nM) was added to a buffer [0.125 M sucrose, 80 mM potassium acetate, 0.5 mM magnesium acetate, 50 mM Hepes (pH 7.4), 0.5 mM tetracaine (Sigma), and 200 μM cycloheximide (Sigma)] containing about 0.5 to 1.5 mg of microsomal protein per milliliter and incubated at 30°C for 30 minutes. Microsomes were then disrupted with 1 percent Triton X-100, and glycosylated NYTC was affinity purified by a Con A-Sepharose 4B column (Pharmacia) [J. Bischoff and R. Kornfeld, *J. Biol. Chem.* **258**, 7907 (1983)]. Typically, approximately 10 to 20 percent of freshly iodinated NYTC incubated in the presence of microsomes bound to Con A-Sepharose. If the microsomes were initially disrupted by 1 percent Triton X-100, the percentage of NYTC bound to Con A decreased to <1 percent. Less than 1 percent of the radioactive NYTC was bound if microsome glycosylated NYTC samples were treated with Endo H or placed on Con A columns in the presence of excess α-methylmannoside (Sigma).
- For Endo H digestion, peptide samples were dissolved in 0.1 M sodium citrate (50 μl); Endo H (Genzyme), at 20 mU/ml was added and incubated at 37°C for at least 12 hours.
- The GSH/GSSG ratios were measured as described [M. E. Anderson, *Methods Enzymol.* **113**, 548 (1985)].
- More than 90 percent of glycosylated NYTC was retained in microsomes stabilized by 0.5 mM tetracaine (Sigma), which is a membrane stabilizer used to improve the integrity of microsomes [G. Scheele, *ibid.* **96**, 94 (1983)].
- CRL-1606 cells (viability >90 percent) were harvested and resuspended (1 × 10<sup>7</sup> cells per milliliter) in fresh Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (2 percent), 37 mM glucose, 6 mM glutamine, and 25 mM Hepes (pH 7.4). Reduced <sup>125</sup>I-labeled NYTC (~10 nM; ~20 μCi/ml) was added, and the cultures were incubated for 2.5 hours at 37°C. Cells were then harvested by silicone oil layer centrifugation (SOLC). Cell samples were separated from the lysis-quenching solution by a layer of silicone oil (AR200, Fluka Chemie AG) of intermediate density in 15-ml polypropylene tubes; during centrifugation at 6000g at 4°C for 10 minutes, only cells penetrated the oil layer and became lysed in the lysis-quenching solution. The lysis-quenching solution varied, depending on the measurements; in all cases, this solution was a strong acid that served to lyse cells, precipitate proteins, and quench thiol interchange simultaneously. For the measurement of secretory pathway redox state, 10 percent TFA, at 1.08 g/ml (with D<sub>2</sub>O to adjust density) was used; for measurement of intracellular GSH/GSSG ratios, 10 percent sulfosalicylic acid (Sigma), 5 percent Triton X-100, 5 mM EDTA (Mallinckrodt), 5 mM DTPA (diethylenetriaminepentaacetic acid; Sigma), at 1.05 g/ml was used. [TFA (10 percent) precipitates proteins as effectively as does SSA (20 percent) or TFA (25 percent) (20)]. The SOLC tubes were chilled at 4°C. After SOLC, 200 mM N-ethylmaleimide (NEM) (20 μl) was added to the cell extract with <sup>125</sup>I-labeled NYTC, and samples were concentrated by centrifugation at reduced pressure. Before they dried, the samples were resuspended in deionized water (200 μl) and the centrifugation at reduced pressure was repeated; this step was again repeated to evaporate as much TFA as possible. Subsequently, 50 mM tris (pH 8.5) (20 μl) was added to the nearly dried cell extracts, and the pH was adjusted to between 7 and 8.5 to initiate sulfhydryl derivatization by NEM. The glycosylated NYTC was then affinity-purified by Con A-Sepharose chromatography and adsorption to a SEP PAC C-18 cartridge. After concentration by centrifugation, peptide samples were treated with Endo H. Endo H-sensitive peptides were purified on SEP PAC C-18 cartridges, centrifuged at reduced pressure, and analyzed by HPTLC. This peptide processing protocol did not result in significant change of redox state of a control peptide. The measurement of intracellular GSH/GSSG ratios was checked by successful recovery of known concentrations of GSH and GSSG from cell extracts.
- <sup>125</sup>I-labeled NYTC was analyzed on Pharmacia PhastSystem. Samples were spotted onto Phast-Gel IEF 3-9 (Pharmacia) along with pI standards (Bio-Rad) and glycosylated, Endo H-treated <sup>125</sup>I-labeled NYTC mixed-disulfide standards (35).
- Cells (~2 × 10<sup>7</sup>) were centrifuged and resuspended in 0.3 ml of water; TFA (50 μl), 100 mM DTT (0.38 μl), and 1 M tris (pH 8.5) (10 μl) were added. Protein precipitates were removed, the sample was centrifuged at reduced pressure and resuspended in water (20 μl) with the pH adjusted to 8.5 with triethylamine. The mixture was reduced by DTT by heating at 60°C for 1 hour; then 100 mM monobromobimane (12 μl) (Calbiochem) was added to derivatize all sulfhydryls, and held for 10 minutes at room temperature in the dark. TFA was then added as a preservative. The samples were analyzed by HPLC as described [R. C. Fahey and G. L. Newton, *Methods Enzymol.* **143**, 85 (1987)].
- For measurement of intracellular GSH/GSSG ratios, cells (1 to 2 × 10<sup>7</sup>) were harvested with SOLC (15). A small portion of the extract was reserved for total glutathione (GSH and GSSG) assay and the remainder was treated with 2-vinylpyridine (Aldrich) and assayed for GSSG. To derivatize GSH, oxygen was purged from the lysate by vortexing under a constant argon jet until all solutions were added. The 2-vinylpyridine (40 μl) was added to 0.5 ml of cell lysate and 95 μl of 3 M potassium phosphate (Sigma) was added dropwise to increase the pH to ~6 and initiate sulfhydryl derivatization of the cell lysate. The tubes were then filled with argon, capped, and agitated for another 2 hours. All GSH and GSSG standards received the same treatment as the cell extracts; all measurements were done in triplicate; and all experiments were sampled in duplicates.
- The redox potentials were calculated by the Nernst equation from the measured GSH/GSSG ratios and the total glutathione concentration
 
$$E_n = E^0 + 2.303 \cdot RT/nF \cdot \log([GSSG]/[GS^-]^-)$$
 where E<sub>n</sub> is the redox potential referred to the normal hydrogen electrode, E<sup>0</sup> is the standard potential of glutathione, -0.24 V [I. M. Torchinskii, *Sulphur in Proteins* (Oxford, New York, 1981), R is the gas constant (8.31 J/deg-mol), T is the absolute temperature (°K), n is the number of electrons transferred (2 for SH-SS exchanges), and F is the Faraday constant (96,406 J/V). The measured intracellular glutathione concentration of CRL-1606 cells was 8.0 ± 1.7 mM; cell volume was calculated from cell size distribution analyzed on a Coulter Z<sub>e</sub> electronic particle counter and channelizer (Coulter Electronics).
- C. Hwang, unpublished results.
- A. Holmgren, *Eur. J. Biochem.* **6**, 475 (1968).
- D. Dolphin, O. Avaramovic, R. Poulson, *Glutathione: Chemical, Biochemical, and Medical Aspects* (Wiley, New York, 1989).
- R. Nilsson, E. Peterson, G. Dallner, *J. Cell Biol.* **56**, 762 (1973).
- Y. Aniya and M. W. Anders, *Arch. Biochem. Biophys.* **270**, 330 (1989).
- J. Martensson, J. C. K. Lai, A. Meister, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7185 (1990); K. Kurosawa *et al.*, *Biochem. Biophys. Res. Commun.* **167**, 367 (1990).
- Prepared canine pancreatic microsomes were shown to be intact and not leaky; they protected *in vitro*-translated and -translocated proteins from protease digestion (20). They also retained glycosylated NYTC (14).
- [<sup>35</sup>S]GSH and [<sup>35</sup>S]cysteine were obtained (NEN, Dupont) in solutions containing DTT, and were HPLC-purified to remove excess DTT. The DTT-free [<sup>35</sup>S]GSH and [<sup>35</sup>S]cysteine were lyophilized and stored in 0.1 percent TFA in water at -75°C.

- For the preparation of [<sup>35</sup>S]GSSG and [<sup>35</sup>S]cystine, DTT-free [<sup>35</sup>S]GSH and [<sup>35</sup>S]cysteine were incubated with an excess (at least 10 times the molar concentration) of the corresponding disulfides in 50 mM tris buffer (pH 8.5) at 50°C for at least 2 hours. For transport studies, microsomes were centrifuged to remove DTT through 1 M sucrose, 50 mM Hepes (pH 7.4) (Beckman TL-100 ultracentrifuge 70,000 rpm) at 4°C for 10 minutes. Microsomes were resuspended in transport buffer (0.125 M sucrose, 80 mM potassium acetate, 0.5 mM magnesium acetate, 50 mM Hepes (pH 7.4), 0.5 mM tetracaine (Sigma), and 200 μM cycloheximide (Sigma) and incubated at 30°C for 1 minute before the <sup>35</sup>S-labeled metabolite was added. The amount transported was assayed by pipetting small portions of transport reaction into ice-cold phosphate-buffered saline (PBS) (5 ml), mixed, and filtered through a GF/A glass fiber filter (Whatman) in a filtration device (Hoefer Scientific Instruments). The filters were washed twice with ice-cold PBS (10 ml), and the radioactivity was quantitated in the presence of Budget-Solve scintillation fluid (4 ml) (Research Products International, Inc.). All sampling was done in triplicates. For all transport assays, radioactivity detected after 10 seconds of transport (in duplicate) was subtracted to account for nonspecific adsorption to the microsomes and the filters.
28. <sup>35</sup>S-Labeled disulfides were incubated with microsomes for 5 minutes, the microsomes were filtered and washed on glass fiber (GF/A) filters. Ice-cold 10 percent (w/v) SSA (5 ml) was added to the filters. After 2 minutes, filters were rinsed twice with ice-cold 5 percent SSA and once with ice-cold PBS. The radioactivity on these filters corresponded to SSA-precipitable radioactivity.
  29. N. Lambert and R. B. Freedman, *Biochem. J.* 213, 235 (1983).
  30. D. M. Ziegler, *Annu. Rev. Biochem.* 54, 305 (1985); R. N. Ondarza, *Biosci. Rep.* 9, 593 (1989); I. A. Cotgreave, L. Atzori, P. Moldeus, in *Sulfur-Containing Drugs and Related Organic Compounds. Chemistry, Biochemistry, and Toxicology. Analytical, Biochemical, and Toxicological Aspects of Sulphur Xenobiochemistry*. L. A. Damani, Ed. (Wiley, New York, 1989), vol. 2, part B, pp. 101–119.
  31. D. M. Ziegler and L. L. Poulsen, *Trends Biochem. Sci.* 2, 79 (1977); J. Issacs and F. Binkley, *Biochim. Biophys. Acta* 497, 192 (1977).
  32. C. Vermeer, *Biochem. J.* 266, 625 (1990).
  33. V. G. Janolino and H. E. Swaisgood, *Arch. Biochem. Biophys.* 258, 265 (1987).
  34. The HPTLC system used for the analysis of GSH/GSSG and cysteine/cystine ratios was similar to that used for the analysis of NYTC redox state, except that the solvent system used was butanol, acetic acid, and water (3:2:2).
  35. <sup>125</sup>I-labeled, glycosylated, Endo H-treated disulfide-linked NYTC's were prepared by incubating reduced, microsome glycosylated, Endo H-treated NYTC with an excess (10,000 times of disulfide in 100 mM tris buffer (pH 8.5) for 1 hour at 50°C. These disulfide-linked NYTCs were then purified on SEP PAC C-18 cartridges.
  36. Samples used for the measurement of GSH/GSSG ratios were first acid-quenched in 0.05 N HCl, and then sulfhydryl groups were derivatized with an excess (2000 times; molar basis) of 2-vinylpyridine (Aldrich) at pH 5.5 for 2 hours. NYTC was treated with NEM, pH5; and the derivative was stored at pH 8.5.
  37. For RNase treatment, microsome samples were incubated with 15 mM EDTA and RNase (200 μg/ml) (Boehringer Mannheim) for 10 minutes on ice. Microsomes were centrifuged (60,000 rpm, 10 minutes, 0.7 M sucrose cushion, Beckman TL-100), resuspended gently in transport buffer, and layered on a sucrose gradient consisting of 0.5 ml each of 26, 31, 34, 36, 38, 40, 42, 44, 46, 48, 50, and 51 percent sucrose solutions (w/v) in D<sub>2</sub>O and 50 mM Hepes (pH 7.4). After centrifugation at 4°C for 3 hours (SW 50 Beckman rotor) at 45,000 rpm, the gradients were fractionated. The radioactivity, protein concentration, sucrose concentration, and α-glucosidase activity of each fraction were quantitated as described [L. Wikström and H. F. Lodish, *J. Cell Biol.* 113, 997 (1991)].
  38. Digestion was done in the presence of proteinase K (20 μg/ml) (Boehringer Mannheim) and 2 mM CaCl<sub>2</sub> for 15 min at 30°C. After digestion, PMSF (2 mg/ml) was added to the mixture and incubation was continued for 5 min on ice.
  39. Samples separated under nonreducing conditions were treated with 50 mM NEM at pH 7.4 and mixed with 2× SDS-PAGE sample buffer and boiled for 5 minutes, and subjected to electrophoresis, which was done as described [U. K. Laemmli, *Nature* 227, 680 (1970)].
  40. Microsomes were sedimented through a layer of silicone oil (0.12 ml) (AR200, Fluka Chemie AG) into 10 percent TFA (70 μl) (density = 1.08 mg/ml) in a total volume of 0.2 ml (TLA-100 rotor, a Beckman TL-100 ultracentrifuge, 100,000 rpm, 4°C). After centrifugation, 200 mM NEM (2 μl) was added to a portion (10 μl) of the supernatant. After 10 minutes at room temperature the mixture was quenched in 10 percent TFA. The centrifuge tubes were then frozen in liquid nitrogen and the bottom layers, which contained the microsome extracts, were cut off and the extracts were recovered. Once the microsome extracts were freed of silicone oil and protein precipitates, 200 mM NEM (20 μl) was added, and TFA was evaporated by centrifugation at reduced pressure (15). Subsequently, 50 mM tris (pH 8.5) (20 μl) was added, and the pH was adjusted to between 7 and 8.5 with triethylamine to initiate sulfhydryl derivatization by NEM. After 10 minutes at room temperature, the redox states of the samples were quenched with 10 percent TFA. The redox states of the <sup>35</sup>S-labeled samples were analyzed by HPTLC (37). This method for the measurement of the ratio of <sup>35</sup>S-labeled GSH to <sup>35</sup>S-labeled GSSG ratio is thought to be accurate because the redox states of a <sup>35</sup>S-labeled glutathione sample did not change after it was processed through this sample preparation protocol (20).
  41. We thank P. Kim, P. Matsudaira, S. Watowich, and J. Weissman for comments. Supported by NSF grant CDR 88-03014 MIT Biotechnology Process Engineering Center.

5 March 1992; accepted 10 August 1992