Glutathione Directly Reduces an Oxidoreductase in the Endoplasmic Reticulum of Mammalian Cells*

The formation of disulfide bonds is an essential step in the folding of many glycoproteins and secretory proteins. Non-native disulfide bonds are often formed between incorrect cysteine residues, and thus the cell has dedicated a family of oxidoreductases that are thought to isomerize non-native bonds. For an oxidoreductase to be capable of performing isomerization or reduction reactions, it must be maintained in a reduced state. Here we show that most of the oxidoreductases are predominantly reduced in vivo. Following oxidative stress the oxidoreductases are quickly reduced, demonstrating that a robust reductive pathway is in place in mammalian cells. Using ERp57 as a model we show that the reductive pathway is cytosol-dependent and that the component responsible for the reduction of the oxidoreductases is the low molecular mass thiol glutathione. In addition, ERp57 is not reduced following oxidative stress when inhibitors of glutathione synthesis or glutathione reduction are added to cells. Glutathione directly reduces ERp57 at physiological concentrations in vitro, and biotinylated glutathione forms a mixed disulfide with ERp57 in microsomes. Our results demonstrate that glutathione plays a direct role in the isomerization of disulfide bonds by maintaining the mammalian oxidoreductases in a reduced state.

In 1963 Anfinsen showed that the folding of a protein, ribonuclease, is spontaneous and depends entirely on the primary structure of the protein (1). However, we have since learnt that the formation of disulfide bonds is a rate-limiting step in protein folding and does not always result in the correct pairing of cysteine residues (2). It is therefore not surprising that in the mammalian endoplasmic reticulum (ER) an entire family of oxidoreductases exist including protein-disulfide isomerase (PDI), ERp57, ERp72, P5, and PDI-related protein (PDIR), which is dedicated to forming native disulfide bonds.

Members of the oxidoreductase family are characterized by a functional CXXC motif that shares structural homology with the small redox protein thioredoxin (3, 4). The active site cysteine residues can exist in either an oxidized redox state where the cysteine residues assume the dithiol form. This motif allows the oxidoreductases to catalyze three essential reactions depending on the initial redox state of the enzyme. A disulfide bond may be formed through the oxidation of a substrate protein requiring the oxidoreductase to gain two electrons, non-native disulfide bonds may be shuffled or isomerized with no net exchange of electrons, or incorrect pairings may be broken or reduced, requiring the oxidoreductase to donate two electrons to the substrate. Therefore, for an oxidoreductase to be capable of forming a disulfide bond, it must itself be oxidized to be capable of accepting electrons. Conversely, an oxidoreductase can only be functional as an isomerase or reductase when it is in a reduced form, to be capable of donating electrons. After each cycle of reduction or oxidation, the oxidoreductase must be returned back to its original, active redox state before it can fulfill further catalytic rounds. Thus, there is a requirement for oxidative and reductive pathways within the cell.

The oxidative pathway for disulfide bond formation has been well characterized where PDI is known to oxidize substrate proteins and is itself maintained in an oxidized form by Ero1 (5, 6), for which the ultimate electron acceptor can be molecular oxygen (7). However, other oxidoreductases such as ERp57 are thought to act as reductases or isomerases and must therefore be maintained in a reduced state to remain active. A candidate for the reduction of the oxidoreductases is reduced GSH. However, the glutathione buffer in the ER is found at a GSH:GSSG ratio of between 1:1 and 3:1; far more oxidizing than that in the cytosol, where it is thought to be as high as 100:1 (8). In addition, it has recently been shown that up to 50% of the glutathione present in the ER is present as mixed disulfides with protein, which had previously gone undetected, demonstrating that glutathione in the ER is even more oxidizing than was formerly thought (9). Before the discovery that Ero1 oxidizes PDI, GSSG was commonly thought to provide oxidizing equivalents for the formation of disulfide bonds. However, it has been demonstrated that protein folding in yeast is more readily compromised by the addition of an oxidant when glutathione synthesis is compromised, suggesting that GSH could still play a role in the reductive pathway (10).

In comparison, specific enzymes have also been discovered that maintain the active redox state of the bacterial oxidoreductases. DsbA oxidizes substrate proteins and is maintained in an oxidized state by DsbB (11). This parallels the way in which PDI oxidizes substrate proteins in eukaryotic cells and is itself oxidized by Ero1. These systems are strikingly similar; DsbA mutants are complemented by the addition of human PDI into the bacterial periplasm (11, 12). The bacterial pathway for the reduction of disulfide bonds is kinetically separate from the oxidation pathway and consists of DsbC and DsbG, which isomerize and reduce substrate proteins and are maintained in a reduced state by DsbD (13). DsbD functions by...
transferring electrons from cytoplasmic thioredoxin to DsbC and DsbG (13–15). This is the sole function of DsbD and gives the similarities between the prokaryotic and eukaryotic oxidative pathways, it has often been speculated that pathways parallel to the prokaryotic oxidative pathway may also exist in eukaryotic cells.

To uncover a reducing pathway in eukaryotic cells we first examined the redox state of a number of oxidoreductases in vivo by modification of free cysteines with the alkylating agent AMS. We show that most of the oxidoreductases exist in a predominantly reduced form in vivo, suggesting that they might act as isomerasers or reductases. Following oxidative stress, we demonstrate that a model protein, ERp57, is quickly reduced, demonstrating that a pathway is in place to maintain the oxidoreductases in a reduced state. However, following oxidation, ERp57 is not reduced in microsomes unless cytosol is present. We show that the cytosolic component capable of reducing ERp57 is GSH, which in turn is reduced by cytosolic glutathione reductase. Inhibition of either glutathione reductase of glutathione synthesis prevents the reduction of ERp57 following oxidation. In addition, following oxidative stress glutathione is reduced by the cell in a time scale compatible with that of ERp57. We also demonstrate that glutathione rapidly reduces ERp57 at physiological concentrations in vitro, and biotinylated glutathione forms a mixed disulfide with ERp57 in microsomes. These findings define a clear role for glutathione in the reductive pathway for the formation of native disulfide bonds, a role that exceeds that of a buffer against hyperoxidation.

**MATERIALS AND METHODS**

**Antibodies—**Antibodies used in this study were raised as described previously (PDI) (16), or were raised in rabbits to the following peptides: DIIDLSDVELDDLGKDEL (P5), IQEEKPKKKKKAQEDL (ERp57), TNYRALREGDERLGKK (PDIR), and KPIEHEATKLSTKKEEL (ERp72).

**Reduction of Redox State in Vivo—**HT1080 cells (a human fibrosarcoma cell line) in suspension were either left untreated or treated with 10 mM DTT or 1 mM DPH. The redox state of proteins was determined using AMS to alkylate free thiols. The redox state in vivo was trapped by alkylation of free thiols with 25 mM N-ethylmaleimide (NEM), and the cells were lysed in 50 mM Tris-HCl containing 150 mM NaCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1% (v/v) Triton X-100 (lysis buffer). The lysis was denatured by boiling for 2 min in the presence of 1% (v/v) SDS. Triton-2-carboxyethylphosphine (TCEP) was added (10 mM) to break existing disulfide bonds, which were then alkylated by the addition of 30 mM AMS (Molecular Probes, Leiden, The Netherlands) for 1 h at room temperature. The lysates were separated by SDS-PAGE and Western blotted to detect various oxidoreductases; oxidized proteins displayed a decrease in mobility.

The reduction of ERp57 was examined following oxidation of HT1080 cells with 0.5 mM DPH. Prior to the experiment the cells were either left untreated or treated with 0.5 mM BSO overnight or 0.1 mM carmustine for 5 h. Samples were retained to show the redox state of ERp57 before oxidation. The cells were incubated with 0.5 mM DPH for 5 min at room temperature. DPH was removed by centrifugation, and the cells were resuspended in fresh buffer containing the appropriate inhibitors at the aforesaid concentrations. At various time points the redox state was trapped by the addition of 25 mM NEM, and the redox state was determined as above.

**Reduction of ERp57 in Microsomes—**Cytosol was prepared following homogenization of 5 × 10⁶ HT1080 cells in 4 volumes of 50 mM Tris-HCl buffer, pH 7.4, 0.25 mM sucrose, 25 mM KCl, 0.5 mM MgCl₂, 1 mM EDTA (Buffer A). The cell homogenates were centrifuged at 150,000 × g to pellet membranes. Recovered cytosol was separated on a PD10 column previously equilibrated with buffer A and eluted in the same buffer. The fractions were monitored for protein concentration (17) and glutathione concentration (see later) and pooled accordingly.

Microsomes prepared from HT1080 cells (18) were either left untreated or treated with 0.5 mM DPH for 5 min. DPH was removed by centrifugation, and oxidized microsomes were incubated in either buffer A or isolated cytosol. At various times, the redox state was determined through modification with AMS as described above.

**Glutathione Assay—**Total glutathione concentration was measured essentially as described previously (19). The cells were lysed in 8 mM HCl, 1.3% (w/v) 5-sulfosalicylic acid on ice for 1 h. The proteins were removed by centrifugation. The acidified sample was added to 0.2 mM NADPH, 0.6 mM Ellman’s reagent, glutathione reductase (1–2 units/ml; tris-phosphate buffer, pH 7.4). The rate of change of absorption at 405 nm was measured over 1 min with respect to GSH standards. To measure oxidized glutathione concentration acidified samples were treated with 0.3% (v/v) 4-vinlypyridine and 5 mM Tris-HCl buffer, pH 8.8, for 1 h at 20°C. The samples were assayed as above with respect to GSSG standards. GSH concentrations were calculated by subtracting the concentration of GSSG from the total glutathione concentration.

**Determination of Redox State in Vivo—**Escherichia coli strain BL21(DE3)pLysS containing a modified version of pET23b expressing His-tagged ERp57 was a kind gift from Professor S. High (University of Manchester, Manchester, UK). His-tagged ERp57 was purified and dialyzed against 50 mM phosphate buffer, pH 7.4 (20). The redox state of ERp57 in vitro was determined by alkylation with AMS. Disulfide exchange was prevented, and the proteins were precipitated by the addition of 10% (v/v) trichloroacetic acid, 25% (v/v) acetone for 1 h on ice. After centrifugation the pellets were washed twice with cold acetone and resuspended in SDS-PAGE sample buffer (0.25 mM Tris-HCl buffer, pH 6.8 containing 4% (v/v) glycerol and 1% (w/v) SDS). Free thiols were alkylated with 30 mM AMS at room temperature, and the redox state was determined using AMS to alkylate free thiols that were trapped by the addition of 2.5% (v/v) streptavidin-agarose that had previously been blocked with 100× binding capacity of biotin (10 μM) and then washed eight times with lysis buffer. The beads were removed, and the supernatant was incubated with 2.5% (v/v) streptavidin-agarose for 1 h at 4°C. The beads were washed with lysis buffer four times and then resuspended in SDS-PAGE sample buffer containing DTT (50 mM) and boiled for 2 min. The samples were separated by SDS-PAGE and Western blotting for ERp57.

**RESULTS**

**ERp57 Is Maintained in a Reduced State in Vivo—**To assess the requirement for a reductive pathway, we first examined the redox state of several ER oxidoreductases within mammalian cells grown in culture. To evaluate the redox state under physiological conditions, we treated intact cells with the membrane permeable alkylating agent NEM to prevent disulfide exchange and freeze redox status. We then treated cell lysates with a second larger alkylating agent (AMS) that caused a shift in mobility when the protein is separated by SDS-PAGE. NEM alklyation prevented AMS modification of free thiols that were present when the proteins were in the intact cells. The second alklyation step was carried out in the presence of a reducing agent; therefore, proteins that contained disulfide bonds when present in intact cells showed a decreased electrophoretic mobility. When the redox state of the known ER oxidoreductases ERp57, ERp72, P5, PDI-related protein, and PDI was assessed using this approach, a clear decrease in mobility was seen for the proteins from intact cells treated with the oxidizing agent DPH in comparison with cells treated with the reducing agent DTT (Fig. 1A, lanes 1 and 2). When no reducing or oxidizing agent was added to cells, most of the oxidoreductases migrated with the mobility of the reduced protein (Fig. 1A, lane 3), demonstrating that they are predominantly reduced at steady state. The exception was PDI, which ran as a diffuse band, suggesting partial oxidation. These results are consistent with those published previously for ERp57 and PDI in mammalian cells (21) and emphasize that although the ER lumen is optimized for the formation of disulfide bonds in secretory proteins, the active site of these particular enzymes are either partially or completely reduced. Such a reduced state would be required if these proteins are involved in disulfide bond reduction of isomerization. The presence of a reductive pathway should enable the ER oxidoreductases to recover from the addition of...
oxidizing agent to re-establish free thiols. To determine whether this can occur, we treated cells with DPS and then evaluated the redox state of ERp57 at various time points after removal of the oxidizing agent. As can be seen (Fig. 1), ERp57 was partially reduced after 5 min and was completely reduced 15 min following removal of the oxidizing agent. Similar results were obtained for the other oxidoreductases (data not shown), thus establishing that in intact cells a robust reductive pathway exists to maintain these proteins in a reduced state.

Glutathione Is Required for the Reduction of ERp57—To further characterize the reductive pathway, we fractionated cells to determine whether the reductive pathway could function in isolated microsomal vesicles. The ERp57 in isolated microsomes was shown to be reduced and could be oxidized by the addition of DPS (Fig. 2A, upper panel, lanes 1 and 2). However, when the microsomes were isolated and resuspended in buffer in the absence of oxidizing agent, no reduction of ERp57 was observed (Fig. 2A, upper panel). Hence all of the factors required for the reductive pathway are not present in isolated microsomal vesicles. To determine whether we could reconstitute the reductive pathway in microsomal vesicles, we repeated the experiment but this time resuspended the oxidized microsomes in isolated cytosol. Under these conditions reduction of ERp57 occurred but with slightly slower kinetics than in intact cells (Fig. 2A, lower panel). The delay is probably due to the fact that the isolated cytosol is diluted by ~5-fold during preparation. It is clear from these results that factor(s) from the cytosol are required for efficient reduction of ERp57.

To provide information regarding the size of the component facilitating reduction, we fractionated the cytosol by size exclusion chromatography to deplete low molecular mass components (>5000 Da). Depleted cytosol was unable to bring about reduction of microsomal ERp57; however, the low molecular mass fraction was able to partially reduce ERp57 (Fig. 2B). That only partial reduction occurred is likely due to dilution of the active component during fractionation. Components within the cytosol that could contribute to reduction of ER oxidoreductases include low molecular mass molecules such as GSH, NADH, NADPH, cysteine, and cysteamine or proteins such as thioredoxin and glutaredoxin. To identify the component present within the low molecular mass fraction responsible for this reduction, we screened a number of reducing components that are present within cytosol for their ability to reduce ERp57 in the absence of added cytosol and at physiological concentrations (22–25). Of all of the compounds tested, only GSH was able to reduce oxidized ERp57 (Fig. 2C). GSH is maintained in a reduced state within the cytosol by the enzyme glutathione reductase with reducing equivalents coming from NADPH. We therefore tested whether the addition of GSSG with and without NADPH to our depleted cytosol could also bring about the reduction of microsomal ERp57. Only a combination of cytosolic proteins, GSSG, and NADPH was able to efficiently reduce ERp57 (Fig. 2D, lane 7), suggesting that GSH is the active component in the cytosol required for reduction of microsomal ERp57.

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**Fig. 1.** ER oxidoreductases are predominantly reduced at steady state and recover rapidly from oxidative stress. A, HT1080 cells (a human fibrosarcoma cell line) in suspension were either left untreated (lane 3) or treated with DTT (10 mM) (lane 1) or DPS (1 mM) (lane 2). The samples were subjected to modification with AMS, SDS-PAGE, and Western blots probed with antibodies to oxidoreductases. Oxidized proteins displayed a decrease in migration. B, reduction of ERp57 in cells following oxidative stress. HT1080 cells were either left untreated (lane 1) or treated with DPS (0.5 mM) for 5 min (lanes 2–7). DPS was removed, and the cells were resuspended in fresh medium and incubated at 37 °C. At specified times following oxidation, the redox state of ERp57 was determined by alkylation with AMS and Western blotting with an anti-ERp57 antibody.
ERp57. The recovery of reduced ERp57 was abolished when an inhibitor of glutathione reductase (carmustine) (26) was added to the cytosol (Fig. 2D, lane 8), demonstrating that the reduction of GSSG to GSH is required for reconstitution of the reductive pathway. In addition no inhibition of recovery was observed when an inhibitor of thioredoxin reductase (cisplatin) (27) was added to the cytosol (Fig. 2D, lane 9), suggesting that thioredoxin is not involved in the ER reductive pathway.

**Glutathione Reduces ERp57 in Vivo**—Having established that GSH can reduce ERp57 using our microsomal assay, we wanted to determine whether GSH was also involved in the reductive pathway within intact cells. To do this we either depleted the intracellular pool of GSH by inhibiting the initial enzyme in GSH synthesis (γ-glutamylcysteine synthetase) with

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**Fig. 2.** Glutathione is the cytosolic component required for reduction of ERp57 following oxidative stress. The redox state was determined by alkylation with AMS, SDS-PAGE, and Western blotting with an antibody to ERp57. A, microsomes prepared from HT1080 cells were either left untreated (lane 1) or oxidized with DPS (0.5 mM) for 5 min (lanes 2–7). DPS was removed, and oxidized microsomes were incubated in either Buffer A (top panel) or isolated cytosol (lower panel). At various times the redox state was determined. B, microsomes were oxidized with as in A and incubated at 37 °C in the presence of either cytosol (lane 1), cytosol depleted of low molecular mass (MW) components (lane 2), or the fractionated low molecular mass components (lane 3). The redox state of ERp57 was determined after 1 h as described. C, microsomes were oxidized as in A and incubated at 37 °C in buffer A containing various low molecular mass compounds at the concentrations indicated. The redox state of ERp57 was determined after 1 h. D, microsomes were oxidized as in A and incubated at 37 °C for 1 h in buffer A containing depleted cytosol and/or NADPH (1 mM) and/or GSSG (0.5 mM) as indicated. Carmustine (BCNU) (0.1 mM) or cisplatin (0.1 mM) were also included in addition to depleted cytosol, NADPH and GSSG (lanes 8 and 9, respectively). The samples were assayed for redox state as above.
Glutathione Directly Reduces ERp57—To determine whether GSH is able to directly reduce ERp57, we first assessed whether the reduction of ERp57 in microsomes was dependent on the concentration of added GSH. Increasing the added GSH concentration from 1 to 10 mM rapidly increased the rate of reduction of ERp57 (Fig. 4A), consistent with a direct role for GSH in reducing ERp57. We then assessed whether GSH could reduce purified recombinant ERp57. For this experiment we first oxidized the protein with DPS and then removed the excess DPS by gel filtration and followed the reduction of oxidized protein either in the absence or presence of 10 mM GSH (Fig. 4B). Disulfide status was determined at individual time points by first quenching disulfide exchange by acidification followed by treatment with AMS. Hence using this approach a decrease in electrophoretic mobility indicates reduction of disulfide bonds. In the absence of GSH no reduction of purified protein was observed (Fig. 4B, upper panel). However, in the presence of GSH rapid reduction occurred with reduced protein being observed after 6 s and full reduction occurring after 5 min (Fig. 4B, lower panel). The reduced protein migrated as a smear rather than a tight band even when the protein was reduced by DTT (Fig. 4B, lane 1), a likely consequence of the acidification process. Despite this anomaly, it is clear from these results that ERp57 can be directly reduced by GSH and that the kinetics of reduction was equivalent to or even faster than the reduction of ERp57 in cells.

The fact that GSH can reduce ERp57 in vitro still does not prove that GSH directly reduces ERp57 when present in the ER. If GSH does indeed directly reduce oxidized ERp57, then a mixed disulfide intermediate between ERp57 and GSH should be formed as part of the reduction process. To determine whether this intermediate can be formed within the ER, we treated microsomes with DPS to oxidize ERp57 and then added a BioGEE to microsomes during the recovery phase. The BioGEE can pass across the cellular membranes, and therefore the formation of a mixed disulfide would result in the biotinylation of ERp57. To determine whether a mixed disulfide was formed, microsomes were first oxidized and then incubated in the absence of oxidant and the presence of BioGEE for 10 min to allow recovery to occur and then treated with NEM to trap any mixed disulfides formed and to quench excess BioGEE. Biotinylated proteins were isolated on streptavidin-agarose beads, eluted with DTT, and protein-separated by SDS-PAGE. ERp57 was then detected by Western blotting. In the absence of BioGEE no ERp57 was isolated with the streptavidin beads (Fig. 4C, lane 2). However, when recovery took place in the presence of BioGEE, ERp57 was isolated (Fig. 4C, lane 3) from cell lysates. No ERp57 was isolated when the streptavidin beads were blocked with biotin prior to the isolation, demonstrating the specificity of the interaction (Fig. 4C, lane 4). These results demonstrate that the biotinylated reagent acted upon oxidized ERp57 bringing about the formation of a mixed disulfide and strongly suggest that GSH can directly reduce ERp57 in cells.

**DISCUSSION**

The oxidoreductase family is essential for the formation of native disulfide bonds, and these enzymes must be either oxidized to be capable of forming disulfide bonds or reduced to reduce or isomerize non-native disulfides. However, although the oxidative pathway involving Ero1 and PDI has been the focus of much research, a reductive pathway had not been clearly defined until now.

For a number of years it was assumed that GSSG might provide oxidizing equivalents for the formation of disulfide bonds, or inhibit disulfide exchange in the ER when present in excess.
bonds in newly synthesized proteins. However, it has now been shown (10) that the elimination of glutathione from yeast cells results in hyperoxidation of proteins entering the secretory pathway and suppresses a temperature sensitive Ero1 mutant, suggesting that glutathione may provide the reducing equivalents to catalyze the reduction of aberrant disulfide bonds. In mammalian cells it has been demonstrated that an increased level of GSH is needed to balance oxidative folding in semipermeabilized cells overexpressing Ero1Lαs, again indicating that GSH might provide reducing equivalents that counterbalance the oxidizing effects of Ero1 (29). In addition, we have demonstrated recently that although lowering the level of GSH in the cell leads to accelerated folding, it also leads to the formation of non-native disulfide bonds, suggesting that GSH may be required for isomerization (30). However, whether the role of glutathione is to buffer against oxidative stress or as a reducing agent for ER oxidoreductases has not yet been described. Our results clearly demonstrate that GSH functions in a reductive pathway in mammalian cells and participates in oxidative folding by maintaining the ER oxidoreductases in a reduced state. In addition, we show that the cytosol is the source of reduced glutathione, which is imported into the ER and directly reduces the oxidoreductases.

Such a direct role for GSH in the reduction of disulfides within the ER would seem to eliminate the necessity for a separate protein-mediated reductive pathway such as the Dsb/DsbC pathway present in the periplasm of *E. coli* or for the involvement of thioredoxin, which provides electrons for the bacterial reductive pathway. Consequently, the redox conditions within the ER lumen are a balance between reducing equivalents coming from the cytosol as GSH or newly synthesized protein thiols and oxidation of protein thiols by Ero1 (29) or any other potential oxidase (31). The segregation of the oxidative and reductive pathways, therefore, is achieved by the fact that GSH is a poor substrate for Ero1 (7) and that Ero1 can oxidize proteins even at 2.5 mM GSH (32). The importance of GSH in the redox balance is highlighted by the fact that when the *GSH1* gene encoding γ-glutamylcysteine synthase (the enzyme catalyzing the rate-limiting step in glutathione synthesis) is mutated in yeast; the resulting strain is only viable in the presence of DTT. Similarly, homozygous mice deficient in γ-glutamylcysteine synthase are embryonic lethal; however, cell lines isolated from the mutants can grow indefinitely in medium supplemented with N-acetylcysteine, suggesting that it is the reducing property of GSH that is essential and not GSH itself (33). Thus, whereas Ero1 is essential for providing oxidizing equivalents, GSH is essential for providing reducing equivalents in eukaryotic cells.

We have shown that GSH is essential in the formation of native disulfide bonds by maintaining the oxidoreductases in a reduced state. The reduction of ERp57 in oxidized microsomes only occurs in the presence of cytosol or, more specifically, GSH. We demonstrate that when glutathione reductase from cytosol depleted of small molecules is supplemented with NADPH and GSSG, it is able to produce sufficient GSH to bring about the reduction of ERp57. In addition, the inhibition of glutathione reductase with carmustine prevents the reduction of ERp57 in cells, suggesting that cytosolic glutathione reductase is the main provider of reduced glutathione for the ER and hence plays an important role in the reductive pathway. However, this also raises two questions. First, we have shown that GSH and biotinylated GSH are able to cross the ER membrane to reduce ERp57; however, the question of how GSH crosses the ER membrane has been an area of much speculation. Import of GSH could occur by either a specific transporter (34) or through pores in the membrane (35). Second, what is the fate of glutathione reductase? This is a critical question in understanding the role of GSH in the reductive pathway. The question of how glutathione is imported into the ER needs to be answered before we can fully understand the role of GSH in the reductive pathway.

**Fig. 4.** Glutathione forms a mixed disulfide with and directly reduces ERp57. A, microsomes were untreated (lane 1) or oxidized then incubated in GSH (1 mM or 10 mM) (lanes 2–7). The redox state of ERp57 was determined at various times. B, purified His-tagged ERp57 was either reduced with DTT (10 mM) (lane 1) or oxidized with DPS (1 mM) (lane 2). DPS was removed by size exclusion chromatography, and then ERp57 was incubated in either 50 mM phosphate buffer, pH 7.4, minus GSH (upper panel) or plus GSH (10 mM) (lower panel) for various times (lanes 3–7). The redox state of ERp57 was trapped by the addition of 10% (v/v) trichloroacetic acid and the redox state determined by alkylation of free thiols with AMS, separation by SDS-PAGE, and staining with Coomassie Blue. The reduced protein migrated as a smear rather than a tight band even when the protein was reduced by DTT (lane 1), a likely consequence of the acidification process. C, microsomes were oxidized with DPS (0.5 mM) and then incubated with either GSH (2.5 mM) (lane 2) or BioGEE (2.5 mM) (lanes 3 and 4) for 10 min. Disulfide exchange was inhibited with NEM (25 mM). Biotinylated proteins were isolated with streptavidin-agarose (lanes 2 and 3) or streptavidin-agarose blocked with excess biotin (lane 4). The proteins were eluted with DTT (50 mM) and analyzed by SDS-PAGE and Western blotting with an anti-ERp57 antibody.
thione in the lumen of the ER once it has been oxidized to GSSG? Because we have shown that the source of GSH for reduction of the ER oxidoreductases is the cytosol, there are a number of possibilities. First, GSSG may be exported back to the cytosol via a specific or unpreferential transporter where it would be quickly reduced by glutathione reductase. A small proportion of GSSG may be reduced by free thiols in nascent polypeptides because they are translocated into the ER lumen; however, this proportion appears not to be significant because ERp57 is not reduced in cells following oxidative stress when glutathione reductase is inhibited. In summary, we have shown that glutathione is reduced in the cytosol and transported into the ER; however, much is still unknown about the details of the trafficking of glutathione in eukaryotic cells.

It has recently been shown that a large proportion of ER luminal glutathione is found as mixed disulfides with proteins (9); hence GSH could reduce substrate proteins directly. Our results show that biotinylated GSH forms a mixed disulfide with ERp57, an observation supported by the fact that glutathione forms mixed disulfides with ERp57 in intact cells (36). Therefore, GSH may either reduce proteins directly or indirectly following reduction of the ER oxidoreductases. However, there is much evidence to suggest that the preferential mechanism for the reduction or isomerization of substrate proteins occurs via the oxidoreductases. First, many substrate proteins are able to fold spontaneously in a glutathione buffer in the absence of oxidoreductases. However, the folding rate is dramatically increased in the presence of enzymes such as PDI (37). For GSH to reduce a non-native disulfide bond, one molecule of GSH must become deprotonated and nucleophilically attack the sulfur of one of the cysteine residues. This produces a glutathionylated protein and for the glutathione molecule to be released, a second deprotonated GSH must nucleophilically attack the sulfur atom of the first glutathione molecule, thereby forming glutathione disulfide and a reduced substrate protein. In contrast, reduction by the oxidoreductases is more efficient because for the second nucleophilic attack the C-terminal cysteine is positioned perfectly to resolve the mixed disulfide. Therefore, the CXXC motif makes reduction or isomerization of substrate proteins via the oxidoreductases highly efficient.

In addition to a kinetic advantage, the reduction or isomerization of substrate proteins via the oxidoreductases has the advantage that the enzymes favor the formation of native over non-native disulfides. For example, PDI isomerizes a scrambled (non-native) substrate more efficiently than a partially reduced (native) substrate (38), and recombinant ERp57 specifically reduces partially folded major histocompatibility complex class I molecules in vitro, whereas it has little effect on folded molecules. The fact that native disulfide bonds are thermodynamically more stable and solvent-inaccessible than non-native disulfides could explain this discrepancy. However, PDI is a known polypeptide-binding protein (39) and displays both chaperone and redox activity during the folding of substrate proteins, where a PDI variant of lipopolysaccharide is observed to increase the yield of correctly folded substrate without increasing the rate of folding (40). In addition PDI, which contains a peptide-binding domain (40), is better able to isomerize kinetically trapped folding intermediates and catalyze disulfide formation in intermediates that have become more compact, compared with thioredoxin, which does not contain a peptide-binding domain (41). Therefore, it is highly advantageous for glutathione to act via the oxidoreductases during the manipulation of disulfides.

The finding that there is a robust pathway for the reduction of the oxidoreductases and the fact that the majority of the oxidoreductases are in a reduced state highlight the importance of the reduction and isomerization pathways in the cell. However, it also raises the question of why so many homologues are needed to fulfill these functions, a role that at first glance could potentially be fulfilled by PDI alone. ERp57 is known to function in association with calnexin and calreticulin (42); however, the roles of many other more recently characterized oxidoreductases are unknown. It is clear that the formation of native disulfide bonds is a highly regulated process potentially involving many enzymes or chaperones, where glutathione plays a central role donating electrons to maintain the oxidoreductases in a reduced, active state.

Acknowledgment—We thank Stephen High for critical reading of the manuscript.

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