A Major Fraction of Endoplasmic Reticulum-located Glutathione Is Present as Mixed Disulfides with Protein*

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Rosemary Bass‡, Lloyd W. Ruddock§, Peter Klappa, and Robert B. Freedman∥
From the Department of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom

The tripeptide glutathione is the most abundant thiol/disulfide component of the eukaryotic cell and is known to be present in the endoplasmic reticulum lumen. Accordingly, the thiol/disulfide redox status of the endoplasmic reticulum lumen is defined by the status of glutathione, and it has been assumed that reduced and oxidized glutathione form the principal redox buffer. We have determined the distribution of glutathione between different chemical states in rat liver microsomes by labeling with the thiol-specific label monobromobimane and subsequent separation by reversed phase high performance liquid chromatography. More than half of the microsomal glutathione was found to be present in mixed disulfides with protein, the remainder being distributed between the reduced and oxidized forms of glutathione in the ratio of 3:1. The high proportion of the total population of glutathione that was found to be in mixed disulfides with protein has significant implications for the redox state and buffering capacity of the endoplasmic reticulum and, hence, for the formation of disulfide bonds in vivo.

The endoplasmic reticulum (ER) lumen of uni- and multicellular eukaryotes is the compartment in which nascent secretory proteins fold to attain their native structure. The luminal environment favors the maturation of secretory proteins by providing chaperones; for example, immunoglobulin heavy chain-binding protein, folding catalysts such as protein disulfide isomerase (PDI), and an oxidizing environment suitable for native disulfide bond formation. There are numerous studies on different aspects of the molecular chaperones and disulfide isomerases (for examples, see Ref. 1–10), but there have been only limited studies directly concerning the redox environment of the ER lumen. This is because of the difficulties of developing suitable methods for characterizing organelle microenvironments. One landmark study (11) using a probe thiol peptide confirmed that glutathione is the principal redox buffer in the ER lumen and reported that the ratio of reduced (GSH) to oxidized (GSSG) glutathione in the secretary pathway is between 1:1 and 3:1. This is considerably more oxidizing than the cytosolic ratio of 30:1–100:1 (11) and similar to the optimum for in vitro folding of disulfide bond-containing proteins; in most cases refolding in vitro is carried out with [GSH] at −2 mM (range, 1–5 mM) and [GSSG] at −0.5 mM (range 0.1–2 mM) after the initial optimization of oxidative refolding of lysozyme (12).

The redox environment of the ER lumen is of great importance for the production of secretory proteins, and a role for glutathione in maintaining appropriate redox conditions in this compartment has been conjectured for many years (13–14). There is indirect evidence for the involvement of glutathione in protein disulfide bond formation in vivo (15), but some basic questions still require an answer. First, how are net oxidizing equivalents delivered to the ER lumen to maintain the observed oxidizing environment? The study of Hwang et al. (11) proposed that oxidized glutathione (GSSG) is preferentially transported from the cytosol into the ER lumen. However, a recent direct study of glutathione transport into mammalian liver microsomal vesicles (16) indicates that only reduced glutathione (GSH) can be taken up into the ER lumen. This is consistent with the results of Cuozzo and Kaiser (17), who have shown that in yeast the product of the ERO1 gene is a major source responsible for providing oxidizing equivalents to protein thiols and glutathione in the secretary pathway; other evidence suggests that GSSG can be generated in the ER through the action of a flavin-containing monoxygenase (18, 19). The pathway for delivery of oxidizing equivalents to nascent polypeptide chains in the ER lumen has become an area of vigorous study and is discussed further below.

An even more basic question concerns the various chemical forms of glutathione in the ER and their absolute concentrations. The initial work (11) did not determine absolute concentrations, only the GSH-GSSG ratio, assuming equilibration with the probe. It had the further limitation that this ratio was determined for the entire secretary pathway rather than for the ER lumen itself, the only compartment where folding of nascent proteins occurs; it is not clear whether the secretary pathway maintains a uniform oxidizing environment throughout.

Here we describe an assay developed to allow direct investigation of the different forms of glutathione in rat liver microsomes, a well characterized model system for the ER compartment (20). For analysis of glutathione in a complex mixture such as a microsomal vesicle suspension, the specific reaction of monobromobimane (mBBr) with thiols was exploited. mBBr is a membrane-permeant member of the bromobimane family of compounds, which have been widely used to investigate thiols in biological systems (for reviews, see Refs. 21–24). The weakly fluorescent mBBr reacts specifically with thiol groups, producing a highly fluorescent thioether. Various...
experimental conditions were employed to convert glutathione in its various forms to the free thiol form GSH. GSH derivatized with mBBr (GSH-mB) was then separated from other reaction mixture components by an amended version of one of the rpHPLC separation protocols described elsewhere (24) and detected and quantitated by a fluorescence detector. An estimate of the luminal volume of the microsomes was made and used to suggest a possible range for the concentration of glutathione-containing species in the ER lumen.

EXPERIMENTAL PROCEDURES

Reaction of GSH with mBBr—Conditions for reacting GSH with mBBr and analyses of the resulting mixtures were optimized with GSH stock solutions. The protocol used for the reaction of different samples with mBBr combined elements of the methods reported elsewhere (23, 24). Thiol-containing samples were mixed with at least a 1 mm stoichiometric excess of mBBr in 20 mM Trizma (Tris base)-methanesulfonic acid (MSA) (pH 8) buffer. The reaction between the thiol and mBBr was allowed to proceed for a certain period of time (usually 10 min) at 25 °C, after which the reaction was stopped by the addition of acid, either MSA (24) or trichloroacetic acid (23). Samples were then diluted with 10 mM MSA for rpHPLC analysis, routinely by 40–100-fold. Samples were prepared and analyzed in triplicate. Samples which were not analyzed immediately after preparation were stored in the dark at −20 °C.

Numerous controls were performed to validate the methodology, the most important of which were the “reagent-only” (where the thiol-containing species was excluded from the reaction mixture) and “unknown” controls (where the thiols in the reaction mixture were alkylated by reaction with an excess amount of N-ethylmaleimide for 10 min before addition of mBBr). Such controls allowed identification of peaks in the rpHPLC traces that were not due to the product of the reaction of GSH with mBBr (GSH-mB). These controls are more extensively discussed in previous publications (21–24).

Separation of GSH-mB by rpHPLC—rpHPLC was performed using a Waters 600 Controller and a Waters 712 WISP autosampler run by Millennium Chromatography Manager (Millipore UK Ltd., Hertfordshire, UK). A VyDAC C-18 column with 5-μm beads and dimensions of 4.6 × 250 mm preceded by a guard column containing Waters Guard-Pak, C-18 precolumn inserts (300 Å) was employed (Hi-Chrom, Reading, UK). A 40-min separation protocol adapted from those reported elsewhere (21, 22) was used employing the following linear gradients of 0.25% (v/v) acetic acid, NaOH, pH 5.5 (solvent A) and methanol (solvent B) to produce clear peaks due to GSH-mB: 0 min at 15% (v/v) solvent B; 5 min at 15% (v/v) solvent B; 15 min at 23% (v/v) solvent B; 20 min at 23% (v/v) solvent B; 25 min at 100% (v/v) solvent B; 30 min at 15% (v/v) solvent B; 40 min at 15% (v/v) solvent B. Fluorescence Detection—GSH-mB was found to exhibit an emission wavelength of 477 nm and maximum excitation at 394 nm, which is consistent with previously reported work (24). These parameters were used for the detection of GSH-mB by a Waters 470 scanning fluorescence detector with a bandwidth of 18 nm and the detector set to maximum gain and minimum attenuation to ensure sensitive detection.

Preparation of Rat Liver Microsomes—Rat liver microsomes were prepared according to a previously reported method (25) and either used immediately or divided into aliquots and stored at −20 °C unless otherwise stated. Aliquots of rat liver microsomes thawed for use were washed in double-distilled H2O to remove extra-microsomal material and any luminal contents released by freeze-thawing. This was performed by resuspending the microsomes in an excess of double-distilled H2O, centrifuging at maximum speed in a benchtop microcentrifuge for 5 min, and the resulting supernatant. To generate samples in which the GSH-mB represented total glutathione in the microsomes, reaction of microsomes with mBBr was preceded by incubation for 1 h at 25 °C in a final concentration of 0.1 mM diethiothreitol (DTT).

To prepare samples containing GS-mB that would be due to a combination of free GSH and GSSG in the microsomal lumen, samples containing microsomes were trichloroacetic acid-precipitated to remove any protein (and, thus, any glutathione bound to protein i.e. GSSG-protein) before further processing. Trichloroacetic acid-precipitated microsomes were prepared by the addition of 0.07 volumes of 100% (v/v) trichloroacetic acid to a microsome suspension; this was shown to be the minimum amount of acid required to reproducibly and completely precipitate the proteins in a suspension of microsomes. The sample was centrifuged in a microcentrifuge for 5 min, and the resulting supernatant was combined with 4 volumes of 100 mM Trizma (pH 10.5) thus raising the pH of the sample to 8. Between 5 and 50 μl of the resulting sample were incubated with DTT for 1 h and subsequently with mBBr for 10 min as above.

To test whether the glutathione content of stored microsomes could be influenced by externally added glutathione, 4.8 mM GSH and 0.1 mM GSSG were chosen as an approximation of cytosolic levels of glutathione (11). Washed, freeze-thawed rat liver microsomes were mixed with 4.8 mM GSH and 0.1 mM GSSG in transport buffer (125 mM sucrose, 80 mM potassium acetate, 0.5 mM magnesium acetate, 50 mM Hepes-potassium hydroxide, pH 7.4). The reaction mixture was incubated at 37 °C, with agitation for 1 or 23 h. Subsequently the samples were subjected to ultracentrifugation at 100,000 × g at 4 °C for 5 min. The supernatant was discarded, and the microsomal pellet was resuspended in 20 mM Trizma-MSA (pH 8) to restore the initial volume of the microsome suspension before the standard mBBr derivatization was performed as described above.

Estimation of Microsomal Luminal Volume—For experiments on rat liver microsomes, the proportion of the total volume due to luminal volume is required for quantitative expression of glutathione units in concentration. An assay was developed exploiting the free permeability of 2-deoxy-β-[3H]glucose ([3H]DG) across the ER membrane. 5–30 μl aliquots of rat liver microsomes were incubated with 10 μl of [3H]DG ([3H]DG = 31–125 nm, specific activity = 185–740 GBq/mmol, an amount of radioactivity present = 0.37 kBq/μl) in a total reaction volume per sample of 100 μl, in transport buffer. Samples were incubated at 37 °C for 1 h (which was shown to be sufficient for the [3H]DG to reach equilibrium across the ER membrane) and centrifuged at 100,000 × g at 4 °C for 5 min. The resulting supernatants and pellets (which were readily separable from the bulk solution) were analyzed by liquid scintillation counting.

RESULTS

Development of the Analytical Method for Glutathione—The reaction of GSH with mBBr was optimized with stock solutions. The optimum reaction time for the reaction was found to be 10 min in a reaction mixture containing a stoichiometric ratio of GSH:mBBr of at least 1:2. This is in accordance with suggestions made in previous work (24). Similarly, the 20 mM Trizma-MSA (pH 8) buffer used in previous studies was found to produce less background than other formulations tested. It was shown that the addition of mBBr to a reaction mixture of GSSG and DTT produced the amount of GSH-mB predicted from the complete reduction of GSSG by DTT both in the presence and absence of added GSH.

Optimized rpHPLC separation of GS-mB is shown in Fig. 1, in conjunction with controls indicating the specificity of the reaction. In Fig. 1 the GS-mB peak can clearly be seen, but there are other minor peaks or base-line fluctuations observed. Because these are also present in the reagent-only and “unknown” controls, they are due to fluorescence of species that are not a product of the reaction of GSH with mBBr and can be ignored in the quantitative analysis of GSH. Control experiments suggest that these small peaks are most likely due to limited hydrolysis of excess mBBr. The GS-mB standard curve (Fig. 2) is linear from 10 to 175 pmol and shows excellent reproducibility.

Analysis of the Different Forms of Glutathione in Microsomes—Water-washed, freeze-thawed microsomes were used for initial studies on glutathione in microsomes to establish...
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The results indicate that glutathione is present in microsomal mixtures did not affect the reaction of GSH with mBBr (data not shown).

Glutathione was then quantitated in freshly prepared rat liver microsomes. We assumed that glutathione would be present as the free thiol (GSH), as oxidized forms, and in combination with proteins; the sample pretreatments described above were designed accordingly. An example rPHPLC trace is shown in Fig. 3, and titrations of the area of the GS-mB peak with increasing volume of microsomal suspension are shown in Fig. 4. The three data sets refer to microsome samples subjected to different pretreatments. The amount of GS-mB representing total microsomal glutathione (top line, samples pretreated with DTT) is greater than that resulting from derivatization of glutathione not present in a complex with protein (middle line, samples precipitated with trichloroacetic acid to remove protein, before reduction with DTT), which is in turn greater than that representing free GSH (bottom line, no DTT treatment).

The results indicate that glutathione is present in microsomal vesicles as free GSH, in the oxidized state presumably as the disulfide GSSG, and in a form where it is bound to protein in a DTT-reducible state; we take this to be mixed disulfides (protein-SSG).

The roughly linear increase in peak area with sample volume (Fig. 4) allows the ratios between the different forms of glutathione in rat liver microsomes to be determined. Data for various microsomal preparations are presented in Table I.

Estimation of Microsome Lumenal Volume and of Lumenal Glutathione Concentrations—The experiments above allow quantitation of microsomal glutathione in terms of moles per unit volume of microsomal suspension. An estimate of the lumenal volume of microsome suspensions is required to express the data as concentration within the lumenal compartment. This was determined by examining the proportion of [3H]DG associated with microsomal pellets relative to the bulk solution after incubation to equilibrium. That [3H]DG was rapidly taken up by the microsomes could be demonstrated (data not shown). Because of its high water solubility, no [3H]DG should be associated with the hydrophobic membrane phase, so pellet radioactivity should predominantly represent [3H]DG within the lumenal volume. Hence, the ratio of pellet:supernatant radioactivity should correspond to the ratio of lumenal:medium volume. However, it is possible that there was radioactivity in the pellet present in external medium physically entrapped...
between the microsomal vesicles or forming a surface layer of liquid associated with the pellet. The most suitable way found to estimate the amount of $^3$HJDG present in the extra-microsomal volume involves extrapolation of the graph presented in Fig. 5. Because virtually no radioactivity is present in pellet fractions in the absence of microsomes, the line of this graph should theoretically cross the origin. However, the line of the graph extrapolates to a positive value for pellet radioactivity at zero added microsomes. This can be taken as representing radioactivity associated with the pellet but not within the luminal phase, allowing a correction for this factor in the calculation of luminal volume.

When the correction was applied the estimations of the luminal volumes of the microsome suspensions were found to range between 7.0 and 19.3% of the total suspension volume. The variation in the luminal volumes is not surprising; as a final step in microsome preparation, the final pellet of microsome vesicles was resuspended in a minimum volume of buffer, giving considerable batch-to-batch variation.

Using these values for luminal volume the concentration of total glutathione in freshly prepared microsomes was estimated as 9.4 mM ($\pm 33\%$, $n = 3$). The total concentration of glutathione in washed, freeze-thawed microsomes was ~10% that found in the freshly prepared microsomes. This was to be expected because of the potential for leakage during storage, freeze-thawing, and subsequent washing. However, the concentrations of total glutathione in washed, freeze-thawed microsomes that were subsequently incubated in cytosolic levels of glutathione for 1 or 23 h before mBBr derivatization were comparable with the levels found in freshly prepared microsomes, and the ability of washed microsomes to accumulate glutathione selectively on incubation in the presence of glutathione concentrations mimicking cytosolic conditions.

**TABLE I**

| Sample                                      | GSH | GSSG | GSS-protein |
|---------------------------------------------|-----|------|-------------|
| Fresh                                       | 3   | 1    | 5           |
| Washed, freeze-thawed                      | 4   | 1    | 7           |
| Washed, freeze-thawed, Incubated for 1 h in 4.8 mM GSH and 0.1 mM GSSG | 8   | 1    | 2           |
| Washed, freeze-thawed, Incubated for 23 h in 4.8 mM GSH and 0.1 mM GSSG | 13  | 1    | 5           |

**DISCUSSION**

**Microsomes as a Model System**—Microsomal suspensions have been used for many years as experimental models of the endoplasmic reticulum. They retain the selective permeability of the ER and the transverse asymmetry of this membrane system (i.e. they are predominantly “right way out”). However they are clearly the product of a process of disruption and resealing so that their content of soluble small molecules cannot be taken as representing that of the intact ER. Furthermore, there is the possibility of thiol-disulfide interchange and overall oxidation during sample preparation. Various approaches can be taken to limit these dynamic changes, but we omitted them since “application of strategies to prevent oxidation can be expected to have the risk of allowing artifactual reduction and vice versa” (26). Notwithstanding this clear limitation, several of our findings reported here suggest that the microsomes represent a useful system broadly representative of the situation in vivo. These findings include the total concentration of glutathione detected in freshly prepared microsomes, the ratio of GSH to GSSG determined both for fresh microsomes and for extensively washed, stored microsomes, and the ability of washed microsomes to accumulate glutathione selectively on incubation in the presence of glutathione concentrations mimicking cytosolic conditions.

**The Concentration and Chemical State of Glutathione in Isolated Microsomes**—The molar ratio of GSH:GSSG for fresh and freeze-thawed microsomes were 3:1 and 4:1 respectively, similar to that reported previously for the secretory pathway of intact cells based on a quite different, indirect approach (11). This ratio is similar to that shown to be at an optimum for folding of disulfide bond-containing proteins in vitro (12). However, between them free GSH + GSSG accounted for less than 50% of the total glutathione detected, the remainder found in DTT-reducible combination with protein, presumably as mixed disulfides. The involvement of such a large percentage of the total microsomal glutathione pool in mixed disulfides with protein is consistent with earlier studies (27, 28), but it has not been appreciated in recent work. In view of its magnitude, this large additional pool of glutathione is unlikely to represent only mixed disulfides with newly translocated or folding proteins. Furthermore, GSH is thought to be unable to reduce or form mixed disulfides with native disulfide bonds. Hence, we suggest that, due to the oxidizing conditions in the ER, a large proportion of surface-exposed free thiols on ER resident proteins, including luminal content and luminally exposed ER membrane proteins, may exist as mixed disulfides with glutathione. Our results give no indication of whether these are found on a broad range of proteins or whether a subset of proteins exists whose function is to generate a mixed disulfide pool. In either case this would have the net effect of significantly increasing the redox buffering capacity in the ER lumen.

Data on ratios between glutathione species in the luminal compartment are independent of estimates of the volume of this compartment, whereas concentration determinations require such an estimate and, hence, are more prone to error. The method used involved the partition of a soluble probe between luminal and bulk aqueous phases, corrected for any carryover of bulk phase into the microsomal pellet. Any additional artifactual association of the probe with the pellet would lead to an overestimation of the luminal volume and, hence, an underestimation of concentrations in the lumen. Nevertheless, the data generated here are credible.

The overall total glutathione concentration in the lumen of freshly prepared microsomes was estimated to be in the range 6–10 mM, very close to estimates of the total concentration in the cytosol. However the total concentration in frozen, thawed, and washed preparations was less than 1 mM, indicating that extensive losses occurred during the processing of these sam-

**Fig. 5.** The association of $^3$HJDG with rat liver microsomes. Varying amounts of rat liver microsomes were incubated with 3.7 kBq of $^3$HJDG in transport buffer at 37°C. After 1 h samples were centrifuged, and pellet and supernatant fractions were analyzed by liquid scintillation counting. Pellet fractions representing uptake into rat liver microsomes are shown. S.D. are plotted as error bars from triplicate samples.
implices. The fact that the ratios between the different forms of glutathione are essentially the same in the fresh and the stored preparations suggests that, during storage and processing, a small fraction of microsomal vesicles retain their full glutathione content, whereas all glutathione is lost from damaged vesicles. This difference has significant implications for the use of microsomes to study native disulfide bond formation.

To determine whether the loss of glutathione from stored microsomes was reversible, they were incubated for an extended period in a medium containing concentrations of reduced and oxidized glutathione comparable with those in the cytosol. The total glutathione content of washed, freeze-thawed rat liver microsomes was restored to levels seen in the freshly prepared microsomes by incubation in cytosolic levels of glutathione for 1 or 23 h. This concentration was greater than that in the external incubation medium. This result supports the existence of a glutathione transport mechanism in the microsomal membrane. Previous work suggests such a mechanism and produces evidence for preferential concentration of GSSG over GSH in the lumen but has led to contradictory conclusions as to whether this is the result of preferential GSSG transport over GSH in the lumen (11, 16).

The ratios of the different forms of glutathione in these incubated preparations did not correspond to those in fresh microsomes; in particular, there was a smaller proportion of GSS-protein present in the incubated microsomes than in fresh microsomes. The current evidence is consistent with an influx of GSH and its oxidation to GSSG followed by a slower conversion of imported glutathione into mixed disulfides with protein. However, the simple system used here is limited and cannot be taken as a model of the interaction between the lumenal compartment and the cytosol in the intact cell.

**Implications of High Levels of Glutathionylation of ER Proteins**—The significance of our observation that more than half of the glutathione in the ER exists as mixed disulfides with proteins is 3-fold. First, several enzymes have been reported to have their activity modulated by glutathionylation (see Refs. 29–32 as examples). Indeed the formation of a mixed disulfide between glutathione and an active site thiol would be expected to have a dramatic effect on activity. Given the high concentration of oxidized glutathione in the ER it is likely that any surface-exposed thiol will be (reversibly) glutathionylated, as evidenced by our findings that more than half of the glutathione in the ER is in protein mixed disulfides. Hence, ER resident proteins that require an active site thiol must have mechanisms in place to prevent or reverse the reaction. Such a mechanism is found in all members of the PDI family (except Eug1p) via nucleophilic attack of the C-terminal thiol of each active site dithiol on any mixed disulfide formed by the reactive N-terminal cysteine.

Second, there is an implication for the redox state of the ER. Our observations indicate that more than half of the glutathione in the ER exists in a disulfide state either in a mixed disulfide with proteins or in oxidized glutathione. Given the different redox potentials of protein-glutathione mixed disulfides it is not possible to say from our observations what the redox potential of the ER is, but it is clearly more oxidizing than previously thought. Furthermore, the buffering capacity of the ER toward reductive stress is very significantly greater than previously thought. Both of these have an impact on all of the possible functions previously assigned to glutathione.

Third, there is an implication for the mechanism of native disulfide bond formation in the ER. In the ER of multicellular eukaryotes the major catalyst of native disulfide bond formation is thought to be the PDI family of proteins (PDI, PDIp, Erp57, Erp72, P5, and PDIr; for reviews, see Refs. 33 and 34). In addition, the iron-sulfur protein Ero1p (17, 35, 36) and various oxidases (18, 19, 37–39) have also been implicated in delivering redox equivalents for native disulfide bond formation. From in vitro studies it is clear that PDI is a very efficient catalyst of disulfide bond isomerization and that it is likely to be essential and sufficient for this process in vivo. PDI is, however, a relatively poor oxidant, and although it may act as the oxidant for nascent proteins in vivo the question arises as to what then acts as the oxidant of reduced PDI.

For many years it was a reasonable working hypothesis that oxidized glutathione is the immediate source of oxidizing equivalents in vivo, either to directly form mixed disulfides with the protein substrate, hence acting as the direct oxidant, or to reoxidize the oxidative catalyst, e.g. PDI. This model is consistent with in vitro data, which has shown that glutathione (GSH + GSSG) can act as an efficient oxidant for protein disulfide formation and that PDI-catalyzed native disulfide bond formation occurs most efficiently at glutathione concentrations approximating those found in the ER (see Ref. 40 as an example and Refs. 3 and 14). Furthermore, comparative studies on the kinetics of protein folding and disulfide bond formation between (a) defined systems with purified components in vitro, (b) subcellular systems for in vitro translation, translocation, and folding, and (c) intact cells suggested that the presence of PDI plus a glutathione redox buffer was sufficient to account for the rates of folding observed in mammalian cells (14). An in vivo finding directly supporting the involvement of GSSG as an oxidant is that mutant forms of lysozyme are secreted from Saccharomyces cerevisiae with unpaired Cys residues in mixed disulfides with glutathione (15). The levels of glutathione-protein mixed disulfide within the ER lumen detailed here suggest that such conversion of an exposed free thiol to protein-SSG is not exceptional.

However these approaches do not directly analyze the pathway for delivering oxidizing equivalents to nascent proteins in vivo. This has recently been the subject of extensive study in S. cerevisiae with the definition of the ERO1, ERF2, and FMO genes and indications that flavin and iron-sulfur cofactors are involved in the transfer of oxidizing equivalents from oxygen to thiol groups in the ER lumen (17–19, 35, 37, 38). One outcome of this work was the demonstration that glutathione is not an absolute requirement for native disulfide bond formation in the ER of S. cerevisiae (17). The system that was proposed was that the membrane-bound protein, Ero1p, acted as an oxidant for PDI, which then oxidized substrate proteins (41, 42), in the same way that the transmembrane protein DsbB acts as the oxidant of DsbA in the bacterial periplasm, which in turn oxidizes bacterial secretory proteins (43). Furthermore, Ero1p has been shown to be sufficient to act as the sole source of oxidizing equivalents in vitro for the PDI-catalyzed reoxidation of RNase (44).

Although these results elegantly showed that glutathione is not an absolute requirement for secretory protein oxidation in yeast, they do not demonstrate that GSSG is not involved in the normal in vivo oxidation pathway. Indeed, they showed that GSH can compete with proteins as acceptors of oxidizing equivalents from Ero1p and, hence, that Ero1p can act to generate luminal GSSG. It is then an issue of kinetics of what the most efficient pathway is for oxidizing equivalents to flow from Ero1p to PDI and GSH/GSSG to ultimately reach substrate proteins.

From the published in vitro and in vivo data, supported by data presented here on the large amount of GSS-protein present in the ER, we suggest that one physiological in vivo pathway for disulfide bond formation does involve GSSG, the most abundant disulfide oxidant in this compartment. One potential
mechanism would be that, as a protein is translocated into the ER, its free Cys residues rapidly form GSS-protein mixed disulfides. Proteins containing both glutathione mixed disulfides and free thiols are excellent substrates for PDI (45), which catalyzes the isomerization to produce an intramolecular protein disulfide and to regenerate reduced glutathione. This process is then repeated with the subsequent or concomitant isomerization of intramolecular protein substrate disulfides by PDI to generate native disulfide bonds. Helenius and co-worker (46) show that the rate-limiting steps for native disulfide bond formation in vitro are the late, complex, isomerization steps, whereas oxidation is much more rapid. This is consistent with long established in vitro model systems (3, 14, 45). Hence, there is no reason to exclude the possibility that GSSG is present as protein mixed disulfides. Such mixed disulfides may play a role as a glutathione reserve and as a component of a redox buffering system, but they may also play a more active role in the process of native protein disulfide bond formation. However, the results only give information on static concentrations, and much more information is required before conclusions can be drawn on kinetics and pathways in vivo. It is yet to be established whether GSSG and GSS-protein mixed disulfides play a major or a minor role in the pathway of protein disulfide bond formation in the cell; the data presented here make it unlikely that their role is negligible. Nevertheless, the complexities of the system are such that parallel in vivo and in vitro studies and detailed kinetic analyses taking all of the components into consideration (at their physiological concentrations) will be required to provide a complete description of the pathway.

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