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Original Research Communication

Efficient Peroxide-Mediated Oxidative Refolding of a Protein at Physiological pH and Implications for Oxidative Folding in the Endoplasmic Reticulum

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Abstract

The majority of secreted and outer membrane eukaryotic proteins contain disulfide bonds, formed by complex interdependent pathways in the endoplasmic reticulum. The current model for the major route of disulfide formation is the regulated flow of oxidizing equivalents from molecular oxygen to the membrane-associated enzyme Ero1 to protein disulfide isomerase, and hence to substrate proteins. One molecule of hydrogen peroxide is produced by Ero1 per disulfide bond made. This peroxide is usually considered to be a dangerous by-product. Here we show that peroxide, added to a refolding buffer or generated enzymatically *in situ*, results in the efficient refolding of a model protein to the native state. At pH 7.0, the kinetics of obtaining the native folded state are more efficient using peroxide than by the use of a glutathione redox buffer. Disulfide bond formation by peroxide is kinetically favored over oxidation of cysteine to cysteine sulfinic acid and over the oxidation of other amino acids in the proteins such as methionine. Hence, unless peroxides are added in excess, oxidative damage to the folding protein is minimal. Our results offer insights into potential mechanisms for disulfide bond formation *in vivo. Antioxid. Redox Signal.* 11, 963–970.

Introduction

Disulfide bonds are covalent linkages formed between two cysteine residues in proteins, whose primary function is to stabilize the folded structure of the protein. Most secreted and outer membrane proteins, including many high-value proteins targeted by the biotechnology industry, contain disulfide bonds. Since any two cysteine residues in a protein have the potential to form a disulfide bond, the correct formation of native disulfide bonds is not trivial. Hence, native disulfide bond formation is often the rate-limiting step in the folding of proteins *in vitro* and *in vivo* (11, 22).

The pathways for native disulfide bond formation in the endoplasmic reticulum (ER) are complex and not fully understood. The current model for the major route for disulfide bond formation *in vivo* is the highly regulated flow of oxidizing equivalents from molecular oxygen to the sulfhydryl oxidase Ero1 to protein disulfide isomerase (PDI) to substrate proteins (14, 15, 17, 25, 31, 36). However, *in vitro* sulfhydryl oxidases, including Ero1, make one molecule of hydrogen peroxide per disulfide bond made (17, 18). While the extent to which Ero1 generates peroxide *in vivo* is unknown, the current assumption is that the *in vitro* results reflect the *in vivo* situation when molecular oxygen is used as the electron acceptor. The peroxide generated is often regarded as a potentially dangerous by-product of disulfide bond formation that results in oxidative stress to cells. However, peroxide can potentially be used to form disulfide bonds via the formation of a cysteine sulfenic acid intermediate. Indeed there is widespread formation of protein sulfenic acids (8, 28) and protein disulfides (5, 12, 37) *in vivo* upon the exogenous addition of low concentrations of peroxide. Hence hydrogen peroxide produced by sulfhydryl oxidases during disulfide bond formation may itself be involved in oxidative protein folding in the ER. This process in turn could potentially be used *in vitro* for the oxidative refolding of proteins produced in inclusion bodies.

To test this hypothesis hydrogen peroxide was added to reduced glutathione or reduced PDI with both being oxidised to the disulfide state. Peroxide was then added to a reduced refolding protein, or generated *in situ* by the action of glucose

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oxidase on glucose, in the absence of a reductant or isomerase. Our results show that these methods result in the rapid and efficient refolding to the native state for the model protein bovine pancreatic trypsin inhibitor (BPTI). Peroxide-mediated oxidative folding of BPTI was more efficient than oxidation by a glutathione-redox buffer at physiological pH. Furthermore unless the peroxide was in excess, no other oxidative modifications of the refolding protein were observed. These results suggest that the peroxide produced by Ero1 and other sulfhydryl oxidases during disulfide bond formation in the ER may be used productively to make disulfide bonds rather than being a harmful by-product.

Materials and Methods

Human PDI, the W111F mutant of the **a** domain of PDI and BPTI, were purified as described previously (19, 20). Pure reduced BPTI was lyophilized and resuspended into 10 m*M* HCl, pH 2.0 to prevent oxidative refolding. The concentrations of proteins were calculated based on their molar extinction coefficients.

Protein refolding was initiated by the addition of denatured reduced protein to the refolding buffer (0.1 *M* sodium phosphate, 1 m*M* EDTA, pH 7.0). BPTI refolding was carried out at 50 μM. When added, PDI was present at 7 μM, H_2O_2 at 1 m*M*, glucose at 10 m*M*, and glucose oxidase at 2.5 or 50 n*M*. When present, the glutathione buffer was 0.5 m*M* GSSG and 2 m*M* GSH. The folding reaction was stopped by the addition of 1.1 *M* iodoacetamide and BPTI and its folding intermediates were purified with a PepClean C-18 spin column (Pierce, Rockford, IL) before ESI-mass spectrometry analysis (Micromass, Manchester, UK). For reverse phase HPLC analysis, the folding reaction was instead stopped by the addition of $1/10^{th}$ volume of 3 *M* HCl. A μRPC C2/C18 ST 4.6/100 column (Amersham Biosciences, Uppsala, Sweden) pre-equilibrated in buffer A (0.065% trifluoracetic acid (TFA)) was used for analysis of the folding intermediates. The gradient used was 20–30% buffer B over 3 column volumes, 30% buffer B for 1 column volume, 30–31% buffer B over 5 column volumes, 31–45% buffer B over 5 columns volumes, 45–100% buffer B over 3 column volumes; where buffer B is 90% acetonitrile, 0.05% TFA.

The rate of peroxide mediated oxidation of glutathione was determined in a coupled assay by measuring the change in absorbance at 340 nm in 50 m*M* phosphate buffer, pH 7.3, including 0.3 m*M* GSH, 0.5 m*M* EDTA, 0.25 units of yeast glutathione reductase (Sigma, St. Louis, MO), 0.1 m*M* NADPH, and 20 μM PDI when present. The reaction was started by adding 0.3 m*M* of H_2O_2.

The rate of oxidation of the active site of the **a** domain of PDI was determined by the decrease in fluorescence of W35 using a KinTek SF-2004 stopped-flow fluorometer (Austin, TX), excitation 280 nm and band–pass emission >320 nm at 25°C, 10 μM PDI **a** domain W111F mutant with 0.1–2.5 *M* hydrogen peroxide or 1–20 m*M* GSSG.

BPTI activity was determined using inhibition of trypsin activity towards the chromogenic trypsin substrate Nα-benzoyl-DL-arginine-p-nitroanilide (Sigma) using the manufacturer's recommended protocol. The concentration of BPTI was 58% of that of the trypsin and BPTI and trypsin were co-incubated for 1 min before the addition of substrate. Trypsin inhibitory activity was determined in triplicate for each sample from at least two independent samples for each refolding condition.

Results

Peroxide oxidation of GSH and PDI

Disulfide bond formation in folding proteins in the ER can potentially occur via multiple routes, including the introduction of disulfide bonds by GSSG or oxidized PDI. Peroxide can potentially oxidize the C*XX*C active site of PDI or oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG), and the relative rates of these reactions may provide information on whether any of the potential routes for using peroxide could be physiologically relevant. The oxidation of GSH by peroxide can be followed by a coupled assay whereby the GSSG formed is reduced back to GSH by glutathione reductase using NADPH, with a concomitant reduction in absorbance at 340 nm (Fig. 1A). Use of this coupled assay reveals that oxidation of glutathione by peroxide is relatively slow (Fig. 1B), presumably since two intermolecular reactions are required and since the pK_a of GSH (pK_a 9.1) is higher than the typical value for cysteine residues (pK_a 8.3) in proteins. In the presence of catalytic amounts of PDI, the overall rate of glutathione oxidation increases circa threefold. Given the relative concentrations of GSH (0.3 m*M*) and PDI (20 μM) in this assay, this implies that the relative rate of PDI oxidation by peroxide is at least 40-fold faster than that of glutathione.

Peroxide oxidation of the active site of PDI was studied directly by fluorescence studies on the active site of the W111F mutant of the catalytic **a** domain of PDI. This mutant contains only W35, which is juxtaposed to the active site and acts as a useful and direct spectroscopic marker of the redox state of

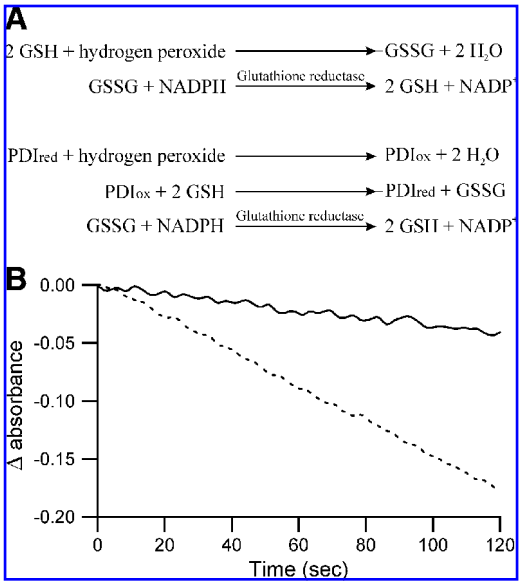

FIG. 1. Coupled assay to examine the rate of oxidation of reduced glutathione by hydrogen peroxide and catalysis by PDI. (**A**) Schematic of the catalyzed and noncatalyzed reactions. (**B**) Representative traces of changes in absorbance due to oxidation of 0.3 m*M* GSH by 0.3 m*M* hydrogen peroxide in the absence (*solid line*) and presence (*dashed line*) of 20 μM human PDI.

the active site (20). When either GSSG or peroxide was added to the reduced protein, a time-dependent decrease in fluorescence was observed, consistent with the difference in fluorescence of the dithiol and disulfide states of the active site (20). The average decrease in fluorescence was $49 \pm 1\%$ for the GSSG-mediated oxidation and $48 \pm 1\%$ for the peroxide-mediated oxidation. Over the concentration range of GSSG tested (1–20 m*M*) the GSSG-dependent oxidation of PDI fitted to a two-step process (data not shown). The first of these steps, the reaction of reduced PDI with GSSG to form a mixed disulfide should be a second order process. However, since GSSG is in large excess over reduced PDI, it will be a pseudo first-order process. The second step, the intramolecular conversion of the PDI-glutathione mixed disulfide to oxidized PDI and GSH will be a first-order process. In line with this mechanism, one of the first-order rate constants obtained in the reaction showed a linear dependence on GSSG concentration and represents the pseudo first-order formation of the GSH–PDI mixed disulfide, with a second-order rate constant of $188\,M^{-1}s^{-1}$. The second rate constant obtained was independent of GSSG concentration, with a first-order rate constant of $0.26\,s^{-1}$, and represents the intramolecular formation of the active site disulfide. These data imply that at physiological concentrations of GSSG the rate limiting step is the nucleophilic attack by the C-terminal active site cysteine on the GSH–PDI mixed disulfide. In contrast, at peroxide concentrations up to 2.5 *M*, the peroxide-dependent oxidation of PDI fitted to a single exponential function (Fig. 2A) and, with peroxide in excess over PDI, the rate constant for this reaction was linearly dependent on the concentration of peroxide. These results are consistent with a pseudo first-order reaction and imply that the rate limiting step for peroxide-mediated oxidation of PDI is the initial formation of the N-terminal cysteine sulfenic acid. Furthermore, the nucleophilic attack by the C-terminal active site cysteine on the sulfenic acid must be significantly $>40\,s^{-1}$ since it is not observed.

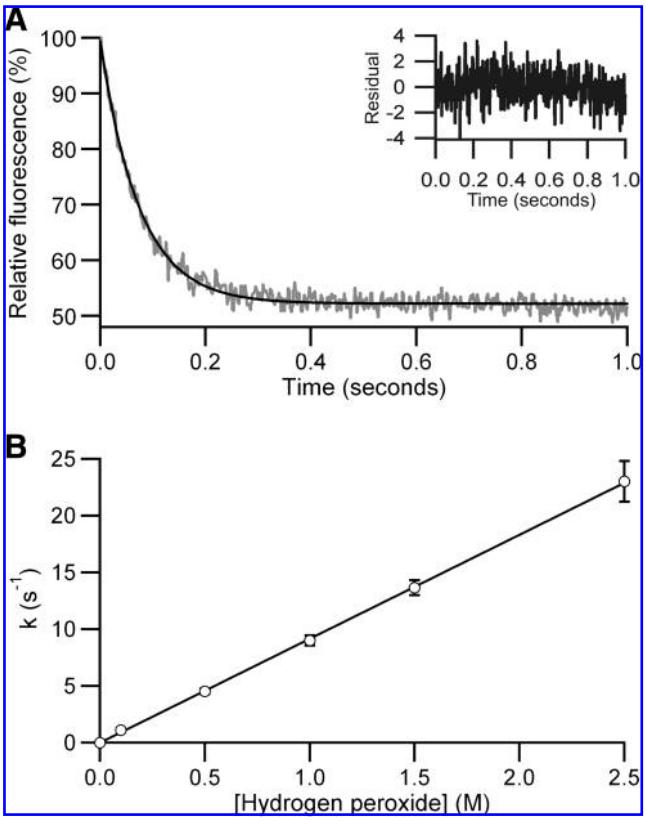

FIG. 2. The kinetics of oxidation of the reduced active site CGHC motif of the W111F mutant of the catalytic a **domain of PDI**. (**A**) Representative trace of the kinetics of oxidation of PDI **a** domain W111F at $10\,\mu M$ in the presence of 1.5 *M* hydrogen peroxide. *Insert* shows the residuals to fitting to a single exponential function. (**B**) Linear dependence of the pseudo first-order rate constant of oxidation on the concentration of hydrogen peroxide ($n = 4$–7). At all concentrations of hydrogen peroxide examined, the kinetics showed random residuals to a single exponential function.

Oxidative protein folding using peroxide

The alternative route for oxidative protein folding using peroxide is direct oxidation of cysteine residues in the folding protein to the disulfide state. The initial step in peroxide mediated disulfide bond formation is the formation of a cysteine sulfenic acid ($-$SOH, reaction 1). The cysteine sulfenic acid can then either react with another molecule of peroxide to form cysteine sulfinic acid (-SO_2H, reaction 3), whose formation prevents subsequent disulfide bond formation, or there can be a nucleophilic attack by another cysteine on the sulfenic acid to form the disulfide (reaction 2).

$$\text{-SH} + H_2O_2 \longrightarrow -\text{SOH} + H_2O \quad (1)$$

$$\text{-SOH} + \text{-SH} \longrightarrow -\text{SS}- + H_2O \quad (2)$$

$$\text{OR} \quad \text{-SOH} + \text{-}H_2O_2 \longrightarrow -SO_2H + H_2O \quad (3)$$

Since the formation of a disulfide bond from the cysteine sulfenic acid in a folding protein is an intramolecular reaction, while the formation of cysteine sulfinic acid is intermolecular, disulfide bond formation would be expected to predominate, providing the peroxide was not in large excess and providing the pH of the reaction was sufficiently high for a significant proportion of the cysteine thiols to be in the nucleophilic thiolate form. Hence, the addition of low concentrations of peroxide at pH values of 7.0 or above should result in the efficient formation of protein disulfide bonds. Since all of the information required to get to the native protein structure is encoded within the primary structure of the protein (1) and hence cysteine residues that form disulfides might be expected to be spatially juxtaposed in the folding intermediates, the use of peroxide potentially could directly form native disulfides in folding proteins.

To test this hypothesis, we used the model protein BPTI. BPTI is a widely studied model of oxidative protein folding. It contains three disulfide bonds in its native state, the folding intermediates are soluble, and in a glutathione redox buffer it gets kinetically trapped in states containing two disulfide bonds (2S) (*e.g.*, see refs. 10 and 39).

When reduced denatured BPTI was added to a glutathione redox buffer at pH 7.0, oxidative refolding occurred, but, as expected, after 2 h the majority of the protein was in the 2S intermediate state, with on average only $\sim$25% having reached the native three disulfide (3S) state (Fig. 3A). Circa 55% more reaches the 3S state over the next 24 h, but the remaining circa 20% is stable in a 2S state for days. In contrast,

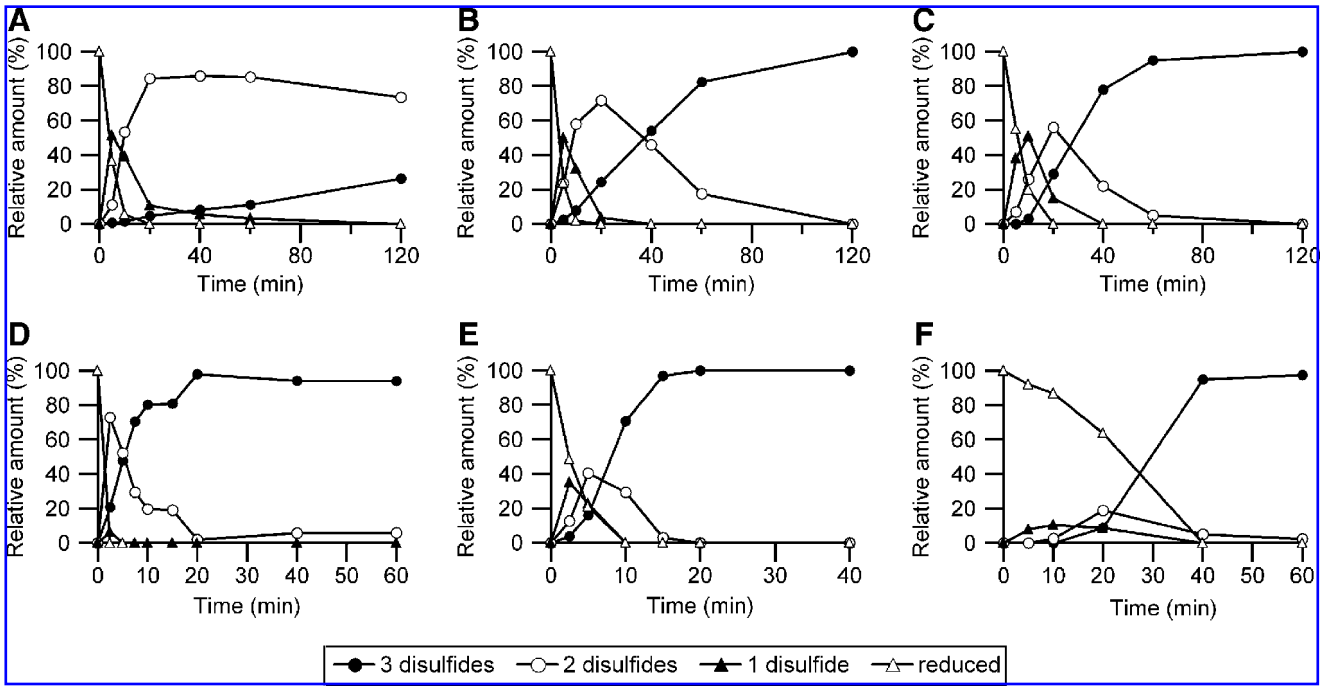

FIG. 3. Representative traces for the kinetics of BPTI oxidative refolding analysed by electrospray mass spectrometry. (**A**) BPTI refolding in a glutathione buffer (2 m*M* GSH and 0.5 m*M* GSSG). (**B**) Refolding using 1 m*M* hydrogen peroxide as the oxidant with no reductant present. (**C**) Refolding using peroxide generated *in situ* by 50 n*M* glucose oxidase. (**D**) Refolding in a glutathione buffer plus catalytic amounts of PDI. (**E**) Refolding using 50 n*M* glucose oxidase plus catalytic PDI. (**F**) Refolding using 2.5 n*M* glucose oxidase plus catalytic PDI. For clarity the glutathionylated intermediates are not shown separately. The sum of all glutathionylated intermediates never represents >10% of the total protein at any timepoint.

2 h after 1 m*M* hydrogen peroxide was added to BPTI only the 3S species was detectable (Fig. 3B). Many of the 2S species formed in a glutathione buffer are kinetically trapped (*e.g.*, see refs. 10 and 39) taking in some cases days to reach the 3S state. However, when the 2S species generated using a glutathione buffer were purified from the glutathione by gel filtration, the subsequent addition of 1 m*M* hydrogen peroxide is able to oxidize these species completely to the 3S state within 1 h (data not shown). This result also implies that for BPTI refolded in a glutathione buffer all of the kinetically trapped 2S species probably contain only native disulfides. This is consistent with the published results of Weissman and Kim (39). Hence peroxides are able to efficiently oxidize dithiols in folding proteins *in vitro*.

While the direct addition of peroxide to a refolding protein is simple, the generation of peroxide *in situ* has the advantage in that it allows the potential for modulating the rate and extent of disulfide bond formation by varying the enzyme or substrate concentrations used to generate it. Glucose oxidase was chosen to generate peroxide *in situ* as it is readily available and already widely used for biotechnological applications. The reaction catalyzed by glucose oxidase is analogous to that catalyzed by Ero1 in the endoplasmic reticulum, with the stoichiometric production of hydrogen peroxide each catalytic cycle.

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{D-gluconate} + H_2O_2$$

$$O_2 + \text{dithiol} \xrightarrow{\text{Erol}} \text{disulfide} + H_2O_2$$

When 50 n*M* glucose oxidase and 10 m*M* glucose was used to generate peroxide, a reaction limited by dissolved oxygen in the buffer, oxidative folding of BPTI occurred quickly and within 1 h on average 98% of 3S species were formed (Fig. 3C).

Oxidized PDI is able to introduce disulfides into folding proteins efficiently, and hence the addition of a catalytic amount of PDI into a glutathione-based refolding buffer increased the rate of BPTI refolding (compare Fig. 3A and D) and prevented the accumulation of kinetically trapped 2S species. Similarly the addition of a catalytic amount of PDI to a glucose oxidase plus glucose peroxide generating system increased the rate of BPTI refolding (compare Fig. 3C and E). Consistent with the relative rates of oxidation of PDI by GSSG and peroxide (see above), the rate of oxidation of BPTI was faster when PDI was combined with a glutathione buffer than with the peroxide generating system.

The glucose oxidase-based peroxide generating system allows the rate of disulfide bond formation to be modulated by varying the amount of glucose oxidase added. Hence, when the glucose oxidase concentration was lowered from 50 to 2.5 n*M*, the initial rate of oxidative folding decreased, but again by 1 h in the presence of catalytic amounts of PDI, 98% of 3S species were formed (Fig. 3F).

Formation of native proteins using peroxide

Peroxides have two potential drawbacks for oxidative refolding. First, they can oxidize other amino acids such as methionine. Methionine is very prone to oxidative modifica-

tion both from mild oxidation agents, including molecular oxygen, and from iodination reactions (32). Met52 of BPTI is prone to oxidation (9) and on average 10% of the BPTI we purify from inclusion bodies and treat with iodoacetamide is a +16 Da species. However, the rate of peroxide-mediated oxidation of other amino acids was slower than that of disulfide bond formation. Even after 2 h in 1 m*M* peroxide the maximal amount of +16 Da species observed in any sample was 21.5% (*i.e.*, 11.5% above basal level). By reducing the time in contact with the excess peroxide, it was possible to reduce the maximal additional oxidative modification to below 10% while allowing the complete formation of 3S species.

Second, oxidation using peroxide alone may result in the formation of non-native disulfide bonds, and without a reductant to help catalyze isomerization, this may result in the final product observed by ESMS having three disulfide bonds, but these being a mixture of native and non-native. To examine this, activity measurements of differently folded BPTI were undertaken. These showed that the 3S BPTI species generated by peroxide were as active as those generated using a glutathione buffer plus PDI with both approaching 100% activity (Fig. 4A). In addition, reverse phase HPLC analysis of BPTI refolded using a glutathione buffer or by peroxide, both in the presence or absence of catalytic amounts of PDI, showed that the majority of the 3S species generated co-eluted as a single peak (see Fig. 4B for example traces), suggesting that they were identical native species. There was however an additional minor peak when peroxide was used (Fig. 4B) which was identified by mass spectrometry as a +16 Da 3S species (data not shown). The activity measurements and reverse phase analysis combined imply that the 3S state obtained in peroxide-mediated oxidative folding is the native disulfide bonded state.

While some oxidative damage was always observed when we used an excess of peroxide, the addition of sub-stoichiometric amounts of peroxide, from 50% to 90% of the amount needed for complete oxidation to disulfides, resulted in disulfide bond formation with no discernable increase in oxidative damage. For example, the addition of 75 μM peroxide to 50 μM BPTI, for 1 h at pH 7.0, resulted in the formation of 10% 3S species, 41% 2S, 39% 1S species, and 10% fully reduced species, with no additional +16 Da species formed during the reaction. This implies a stoichiometric conversion of peroxide to disulfide. It also implies that the reaction of cysteine with peroxide is much faster than the reaction of other amino acids, including methionine with peroxide. This is consistent with published data that the rate of biological thiolates with hydrogen peroxide have second-order rate constants in the range 18–28 $M^{-1}s^{-1}$ (40), while oxidation of methionine residues in human α1-antitrypsin by hydrogen peroxide has second-order rate constants in the range 0.001–0.012 $M^{-1}s^{-1}$ at pH 7 (16). Furthermore, our results imply that the intramolecular reaction of cysteine sulfenic acid in BPTI and PDI with cysteine to form a disulfide bond is much more rapid than that of cysteine sulfenic acid with peroxide, as no detectable cysteine sulfinic acid species were observed by mass spectrometry. The rapidity of the intramolecular reaction of cysteine sulfenic acid with cysteine in BPTI is consistent with the results obtained by fluorescence stopped-flow experiments on the peroxide mediated oxidation of PDI, which imply a half life of the cysteine sulfenic acid state of <17 ms (see above).

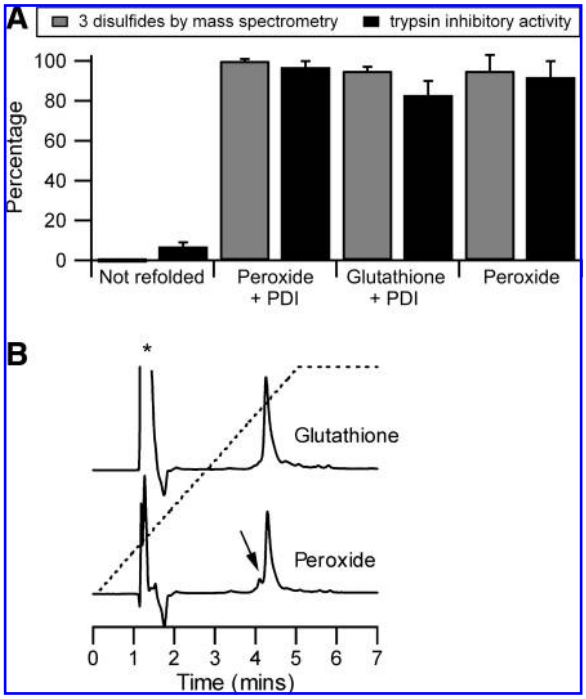

FIG. 4. BPTI refolded using peroxide or a glutathione buffer are essentially identical. (**A**) Comparison of formation of the 3S state of BPTI *versus* the trypsin inhibitory activity of the protein folded by different methods. BPTI was allowed to fold for 2 h in different oxidative systems and then one-half of the sample was processed for mass spectrometric analysis while the other was used for a trypsin inhibitory activity assay. A sample of reduced BPTI that was not refolded acted as a control sample. (**B**) Reverse phase HPLC analysis of the 3S species of BPTI refolded using a glutathione buffer for 5 h and using 1 m*M* peroxide for 1.75 h. Refolding was quenched by the addition of $1/10^{th}$ volume of 3 *M* HCl. The main glutathione folded and peroxide folded peaks co-elute indicated the formation of native BPTI in the peroxide system. The peaks marked * represent buffer components, the *dotted line* the acetonitrile gradient. The additional peak marked with an *arrow* on the peroxide folded material was identified by ESMS as being a +16 Da species and hence represents oxidative modification of the protein (*e.g.*, of methionine residues).

Discussion

Disulfide bond formation in the ER is complex, with multiple possible routes for oxidative protein folding (Fig. 5). The widely accepted major route for dithiol oxidation to a disulfide is Ero1 oxidation of PDI followed by PDI oxidation of substrate proteins. This route, along with potential direct oxidation of folding proteins by other sulfhydryl oxidases, generates one molecule of hydrogen peroxide per disulfide bond made *in vitro* when using molecular oxygen as the terminal electron acceptor (17, 18). This seems to be an inefficient route for oxidation if peroxide is a nonfunctional by-product. It is also a potentially dangerous mechanism since peroxide can cause oxidative damage to proteins, lipids, and other molecules within the ER. Furthermore, since an actively secreting B-cell can make of the order of 100,000 disulfide bonds per second (6), this process would generate milli-molar

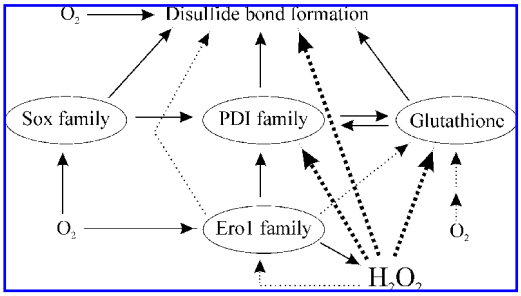

FIG. 5. Pathways for disulfide bond formation in the endoplasmic reticulum. Multiple pathways for dithiol oxidation to a disulfide in a folding protein exist. Direct oxidation by molecular oxygen, while widely used *in vitro*, is too slow to have physiological significance *in vivo*. Whereas members of the sulfhydryl oxidase family are extremely efficient at making disulfide bonds in folding proteins (34, 35) or transferring them to PDI family members (30), the human family member, Qsox, has recently been shown not to be located in the endoplasmic reticulum (7). PDI family members (13) and GSSG can both introduce disulfide bonds into folding proteins, but both need to be reoxidized to complete the catalytic cycle. PDI is thought to be reoxidized by Ero1 (15, 25, 31, 36) while the source of GSSG is under debate in the literature. The hydrogen peroxide made by Ero1 each catalytic cycle (17) has the potential to oxidize dithiols in substrate proteins, to oxidize reduced glutathione to GSSG, to oxidize the active site of PDI family members, and to form the regulatory disulfides (2, 3, 31) in Ero1, and hence shut down peroxide production. All *dotted arrows* represent hypothetical redox routes which have not been demonstrated experimentally *in vivo*. The *bold dotted arrows* indicate routes shown in this work by *in vitro* methods.

amounts of peroxide in the ER every minute, clearly unacceptably high levels given the damage that peroxide can cause, including apoptosis (38), and the use of low levels of peroxide in cellular signalling events (27, 38). Recently data has been published supporting the notion that disulfide bond formation does not cause oxidative stress *in vivo*. In an elegant study, Malhotra and co-workers demonstrated that the accumulation of misfolded proteins in the ER causes oxidative stress and induces apoptosis in cell culture and in mice (21). The same study also showed that overexpression of a protein that contained eight disulfide bonds which efficiently folded in the ER did not lead to the same effects and hence the accumulation of reactive oxygen species was not likely to be a direct consequence of *de novo* disulfide bond formation. Hence cells must have efficient mechanisms in place to remove any peroxide generated by Ero1 during disulfide bond formation *in vivo*.

In other cellular compartments, high levels of peroxide are removed by the action of catalase. However, there are no reports of a catalase isoform being present in the ER, nor can we find a mammalian catalase with an ER-signal sequence by database searching. Since catalase appears to be absent and since peroxide is toxic when present in high amounts, the cell must have other mechanisms in place in the ER to prevent accumulation of peroxides.

While catalase is very efficient at removing peroxides, there are many other enzyme systems which could neutralize the potential harmful effects of the peroxide formed during oxidative protein folding. For example, it has recently been published that there is an ER resident peroxiredoxin, Prx4 (33). Peroxiredoxin family members are widely involved in cytoplasmic processes involving peroxide, including signalling (42). They efficiently reduce peroxide to water, with k_{cat} values of $\sim 50\,s^{-1}$ and in the process a disulfide bond is formed within the peroxiredoxin or between two peroxiredoxin monomers (23, 24, 29, 41, 43). While it has not yet been characterized in detail, Prx4 may therefore be involved in the detoxification of peroxide in the ER via the formation of disulfide bonds. However, this cannot be a general mechanism since many organisms, for example *S.cerevisiae*, appear to lack an ER resident peroxidredoxin. It is also possible that an as yet unidentified redox process may destroy any peroxide formed during oxidative protein folding. However, our hypothesis that peroxides represent a potentially efficient source of oxidizing equivalents to oxidize dithiols to disulfides and hence cells may utilize that potential, seems a simple, logical, and generic solution to the problem. This hypothesis is also supported by the recent identification of two human ER resident glutathione peroxidase family members (26), which appear to interact with Ero1α *in vivo* (unpublished results).

Our *in vitro* refolding results clearly show that peroxides are able to efficiently oxidize dithiols in folding proteins to the native disulfide state at physiological pH. Furthermore, for BPTI this reaction proceeds to the native disulfide bonded state with minimal oxidative side reactions unless the peroxide is in excess. For BPTI the second-order rate constant for the disappearance of the fully reduced species in a glutathione redox buffer ($7.3\,M^{-1}s^{-1}$) is comparable with that in 1 m*M* peroxide ($5.0\,M^{-1}s^{-1}$), with both being orders of magnitude faster than air oxidation. However, there is one clear difference in the final species produced, peroxides drive oxidative refolding to completion within 2 h, while in a glutathione buffer the reaction gets kinetically trapped in 2S species. This difference between the oxidative systems may be due to one of two effects: (a) the small size of peroxide may allow access to buried thiols which are inaccessible to the larger, highly charged oxidized glutathione (GSSG); (b) the nature of the intermediate. The intermediate in the glutathione-based reaction is the glutathionylated protein. Since glutathione is three amino acids in size and highly charged, this may have a significant effect on the local structure of the folding intermediate and may raise the pK_a of spatially adjacent thiol groups decreasing their nucleophilicity and hence their ability to form disulfides. In contrast the intermediate in the peroxide-based reaction is the cysteine sulfenic acid which is only marginally larger than cysteine and is only partially charged at pH 7.0 and hence is less likely to have an effect on the local structure of the folding intermediate. The ability of peroxide to rapidly rescue the kinetically trapped 2S species formed in a glutathione buffer implies that for BPTI the ability to access dithiols that GSSG cannot is probably a factor in the relative ability of peroxide to generate native disulfide bonded proteins.

In addition to the direct oxidation of dithiols in folding proteins, peroxides are also able to oxidize other dithiols to disulfides *in vitro*, including glutathione and the C*XX*C active site of PDI. Since oxidized PDI and GSSG are able to introduce disulfide bonds into folding proteins, all of these oxidation reactions could ultimately have the same net effect *in vivo*, that is, oxidative folding of proteins in the ER (Fig. 5). As well as potentially contributing to disulfide bond formation in fold-

ing proteins, the hydrogen peroxide made by Ero1 in each catalytic cycle has the potential to form the regulatory disulfide bonds (2, 3, 31) in Ero1 family members and hence to shut down both disulfide bond formation and peroxide production. This would provide a simple and elegant regulatory mechanism.

While only *in vitro* data are presented here, the results suggest that peroxide produced by Ero1 may contribute to oxidative folding in the ER, either by directly generating disulfides in folding proteins or more likely via an indirect mechanism such as the oxidation of GSH or of PDI-family members or other proteins such as the ER-resident glutathione peroxidases (26) or Prx4 (33). *In vivo* conformation of our model is difficult. Since the activity of Ero1 family members are highly regulated (2, 3, 31) and the ER contains a large amount of free thiols, we would not expect peroxide to accumulate. Indeed we would suggest that the cell most likely utilizes the peroxide at the point of production rather than allowing it to freely diffuse away. Furthermore, from the *in vitro* kinetics we would not expect cysteine sulfenic acid to accumulate, since the half life of the PDI cysteine sulfenic acid *in vitro* appears to be <17 ms, or for significant amounts of cysteine sulfinic acid to be formed *in vivo* as a by-product of this process. Finally, since a significant proportion of Ero1 in cells is in the inactive state (2, 4) any mechanisms by which peroxide were removed from the system *in vivo* by other means would be unlikely to affect the efficiency of disulfide bond formation, since there is very significant spare capacity in the system. However, the lack of reported experimental data that disulfide bond formation is directly linked to oxidative stress or to the production of oxidative damage such as protein cysteine sulphinic acids in the ER, combined with the recent publication that demonstrates that the overproduction of a protein containing eight disulfide bonds does not cause oxidative stress *in vivo* (21), implies that cells must have an efficient method for dealing with the peroxide produced. Our model, supported by our *in vitro* data, provides an explanation to reconcile published *in vitro* and *in vivo* data with the physiology of professional secretory cells in a manner that is generic for all cell types that possess an ER.

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Abbreviations

BPTI, bovine pancreatic trypsin inhibitor; ER, endoplasmic reticulum; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high pressure liquid chromatography; PDI, protein disulfide isomerase; TFA, trifluoroacetic acid.

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No competing financial interests exist.

References

1. Anfinsen CB. Principles that govern the folding of protein chains. *Science* 181: 223–230, 1973.
2. Appenzeller–Herzog C, Riemer J, Christensen B, Sørensen ES, and Ellgaard L. A novel disulfide switch mechanisms in Ero1α balances ER oxidation in human cells. *EMBO J* 27: 2977–2987, 2008.
3. Baker KM, Chakravarthi S, Langton KP, Sheppard AM, Lu H, and Bulleid NJ. Low reduction potential of Ero1α regulatory disulfides ensures tight control of substrate oxidation. *EMBO J* 27: 2988–2897, 2008.
4. Bertoli G, Simmen T, Anelli T, Molteni SN, Fesce R, and Sitia R. Two conserved cysteine triads in human Ero1 cooperate for efficient disulfide bond formation in the endoplasmic reticulum. *J Biol Chem* 279: 30047–30052, 2004.
5. Brennan JP, Wait R, Begum S, Bell JR, Dunn MJ, and Eaton P. Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J Biol Chem* 279: 41352–41360, 2004.
6. Cenci S and Sitia R. Managing and exploiting stress in the antibody factory. *FEBS Lett* 581: 3652–3657, 2007.
7. Chakravarthi S, Jessop CE, Willer M, Stirling CJ, and Bulleid NJ. Intracellular catalysis of disulfide bond formation by the human sulfhydryl oxidase, QSOX1. *Biochem J* 404: 403–411, 2007.
8. Charles RL, Schroder E, May G, Free P, Gaffney PR, Wait R, Begum S, Heads RJ, and Eaton P. Protein sulfenation as a redox sensor: proteomics studies using a novel biotinylated dimedone analogue. *Mol Cell Proteom* 6: 1473–1484, 2007.
9. Concetti A, Angeletti M, Fioretti E, and Ascoli F. Selective oxidation of methionine residues in Kunitz-type protease inhibitors. *Biol Chem Hoppe Seyler* 370: 723–728, 1989.
10. Creighton TE. Protein folding pathways determined using disulfide bonds. *Bioessays* 14: 195–199, 1992.
11. Creighton TE, Zapun A, and Darby NJ. Mechanisms and catalysts of disulfide bond formation in proteins. *Trends Biotech* 13: 18–23, 1995.
12. Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, and Schubert D. Protein disulfide bond formation in the cytoplasm during oxidative stress. *J Biol Chem* 279: 21749–21758, 2004.
13. Ellgaard L and Ruddock LW. The human protein disulfide isomerase family: substrate interactions and functional properties. *EMBO Rep* 6: 28–32, 2005.
14. Frand AR and Kaiser CA. The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. *Mol Cell* 1: 161–170, 1998.
15. Frand AR and Kaiser CA. Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. *Mol Cell* 4: 469–477, 1999.
16. Griffiths SW and Cooney CL. Relationship between protein structure and methionine oxidation in recombinant human α1-antitrypsin. *Biochemistry* 41: 6245–6252, 2002.
17. Gross E, Sevier CS, Heldman N, Vitu E, Bentzur M, Kaiser CA, Thorpe C, and Fass D. Generating disulfides enzymatically: Reaction products and electron acceptors of the endoplasmic reticulum thiol oxidase Ero1p. *Proc Natl Acad Sci USA* 103: 299–304, 2006.
18. Hoober KL, Joneja B, White HB III, and Thorpe CA. A sulfhydryl oxidase from chicken egg white. *J Biol Chem* 271: 30510–30516, 1996.
19. Karala AR, Psarrakos P, Ruddock LW, and Klappa P. Protein disulfide isomerases from *C. elegans* are equally efficient at thiol-disulfide exchange in simple peptide-based systems but show differences in reactivity towards protein substrates. *Antioxid Redox Signal* 9: 1815–1823, 2007.

20. Lappi AK, Lensink MF, Alanen HI, Salo KE, Lobell M, Juffer AH, and Ruddock LW. A conserved arginine plays a role in the catalytic cycle of the protein disulfide isomerases. *J Mol Biol* 335: 283–295, 2004.
21. Malhotra JD, Miao, H, Zhang K, Wolfson A, Pennathur S, Pipe SW, and Kaufman RJ. Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc Natl Acad Sci USA* 105: 18525–18530, 2008.
22. Molinari M and Helenius A. Glycoproteins form mixed disulfides with oxidoreductases during folding in living cells. *Nature* 402: 90–93, 1999.
23. Parsonage D, Karplus PA, and Poole LB. Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. *Proc Natl Acad Sci USA* 105: 8209–8214, 2008.
24. Peskin AV, Low FM, Paton LN, Maghzal GJ, Hampton MB, and Winterbourn CC. The high reactivity of peroxiredoxin 2 with H_2O_2 is not reflected in its reaction with other oxidants and thiol reagents. *J Biol Chem* 282: 11885–11892, 2007.
25. Pollard MG, Travers KJ, and Weissman JS. Ero1p: A novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. *Mol Cell* 1: 171–182, 1998.
26. Raykhel I, Alanen H, Salo K, Jurvansuu J, Nguyen VD, Latva–Ranta M, and Ruddock L. A molecular specificity code for the three mammalian KDEL receptors. *J Cell Biol* 179: 1193–1204, 2007.
27. Rhee SG. Cell signaling. H_2O_2, a necessary evil for cell signaling. *Science* 312: 1882–1883, 2006.
28. Saurin AT, Neubert H, Brennan JP, and Eaton P. Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. *Proc Natl Acad Sci USA* 101: 17982–17987, 2004.
29. Seo MS, Kang SW, Kim K, Baines IC, Lee TH, and Rhee SG. Identification of a new type of mammalian peroxiredoxin that forms an intramolecular disulfide as a reaction intermediate. *J Biol Chem* 275: 20346–20354, 2000.
30. Sevier CS, Cuozzo JW, Vala A, Aslund F, and Kaiser CA. A flavoprotein oxidase defines a new endoplasmic reticulum pathway for biosynthetic disulfide bond formation. *Nat Cell Biol* 3: 874–882, 2001.
31. Sevier CS, Qu H, Heldman N, Gross E, Fass D, and Kaiser CA. Modulation of cellular disulfide-bond formation and the ER redox environment by feedback regulation of Ero1. *Cell* 129: 333–344, 2007.
32. Shechter Y, Burstein Y, and Patchornik A. Selective oxidation of methionine residues in proteins. *Biochemistry* 14: 4497–4503, 1975.
33. Tavender TJ, Sheppard AM, and Bulleid NJ. Peroxiredoxin IV is an endoplasmic reticulum-localized enzyme forming oligomeric complexes in human cells. *Biochem J* 411: 191–199, 2008.
34. Thorpe C and Coppock DL. Generating disulfides in multicellular organisms: Emerging roles for a new flavoprotein family. *J Biol Chem* 282: 13939–13933, 2007.
35. Thorpe C, Hoober KL, Raje S, Glynn NM, Burnside J, Turi GK, and Coppock DL. Sulfhydryl oxidases: Emerging catalysts of protein disulfide bond formation in eukaryotes. *Arch Biochem Biophys* 405: 1–12, 2002.
36. Tu BP, Ho–Schleyer SC, Travers KJ, and Weissman JS. Biochemical basis of oxidative protein folding in the endoplasmic reticulum. *Science* 290: 1571–1574, 2000.
37. van der Wijk T, Overvoorde J, and den Hertog J. H_2O_2-induced intermolecular disulfide bond formation between receptor protein-tyrosine phosphatases. *J Biol Chem* 279: 44355–44361, 2004.
38. Veal EA, Day AM, and Morgan BA. Hydrogen peroxide sensing and signaling. *Mol Cell* 26: 1–14, 2007.
39. Weissman JS and Kim PS. A kinetic explanation for the rearrangement pathway of BPTI folding. *Nat Struct Biol* 2: 1123–1130, 1995.
40. Winterbourn CC and Metodiewa D. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic Biol Med* 27: 322–328, 1999.
41. Wood ZA, Poole LB, Hantgan RR, and Karplus PA. Dimers to doughnuts: Redox-sensitive oligomerization of 2-cysteine peroxiredoxin. *Biochemistry* 41: 5493–5504, 2002.
42. Wood ZA, Poole LB, and Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300: 650–653, 2003.
43. Yamamoto Y, Ritz D, Planson AG, Jönsson TJ, Faulkner MJ, Boyd D, Beckwith J, and Poole LB. Mutant AhpC peroxiredoxin suppress thiol-disulfide redox deficiencies and acquire deglutathionylation activity. *Mol Cell* 29: 36–45, 2008.

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3. Ester Zito, Henning Gram Hansen, Giles S.H. Yeo, Junichi Fujii, David Ron. 2012. Endoplasmic Reticulum Thiol Oxidase Deficiency Leads to Ascorbic Acid Depletion and Noncanonical Scurvy in Mice. *Molecular Cell* . [CrossRef]
4. Lloyd W. Ruddock . 2012. Low-Molecular-Weight Oxidants Involved in Disulfide Bond Formation. *Antioxidants & Redox Signaling* **16**:10, 1129-1138. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
5. Éva Margittai , Péter Löw , Ibolya Stiller , Alessandra Greco , Jose Manuel Garcia-Manteiga , Niccolo Pengo , Angelo Benedetti , Roberto Sitia , Gábor Bánhegyi . 2012. Production of H2O2 in the Endoplasmic Reticulum Promotes In Vivo Disulfide Bond Formation. *Antioxidants & Redox Signaling* **16**:10, 1088-1099. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
6. Miklós Csala , Éva Kereszturi , József Mandl , Gábor Bánhegyi . 2012. The Endoplasmic Reticulum As the Extracellular Space Inside the Cell: Role in Protein Folding and Glycosylation. *Antioxidants & Redox Signaling* **16**:10, 1100-1108. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
7. Francisco R.M. Laurindo, Luciana A. Pescatore, Denise de Castro Fernandes. 2012. Protein disulfide isomerase in redox cell signaling and homeostasis. *Free Radical Biology and Medicine* **52**:9, 1954-1969. [CrossRef]
8. Taichi Kakihana , Kazuhiro Nagata , Roberto Sitia . 2012. Peroxides and Peroxidases in the Endoplasmic Reticulum: Integrating Redox Homeostasis and Oxidative Folding. *Antioxidants & Redox Signaling* **16**:8, 763-771. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
9. Gábor Bánhegyi , Éva Margittai , András Szarka , József Mandl , Miklós Csala . 2012. Crosstalk and Barriers Between the Electron Carriers of the Endoplasmic Reticulum. *Antioxidants & Redox Signaling* **16**:8, 772-780. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
10. Nicolas Dejeans, Christophe Glorieux, Samuel Guenin, Raphael Beck, Brice Sid, Rejane Rousseau, Bettina Bisig, Philippe Delvenne, Pedro Buc Calderon, Julien Verrax. 2012. Overexpression of GRP94 in breast cancer cells resistant to oxidative stress promotes high levels of cancer cell proliferation and migration: Implications for tumor recurrence. *Free Radical Biology and Medicine* . [CrossRef]
11. Xi Wang, Likun Wang, Xi'e Wang, Fei Sun, Chih-chen Wang. 2011. Structural insights into the peroxidase activity and inactivation of human peroxiredoxin 4. *Biochemical Journal* . [CrossRef]
12. András Szarka, Gábor Bánhegyi. 2011. Oxidative folding: recent developments. *BioMolecular Concepts* ---. [CrossRef]
13. Neil J. Bulleid, Lars Ellgaard. 2011. Multiple ways to make disulfides. *Trends in Biochemical Sciences* . [CrossRef]
14. Anna-Kaisa Lappi, Lloyd W. Ruddock. 2011. Reexamination of the Role of Interplay between Glutathione and Protein Disulfide Isomerase. *Journal of Molecular Biology* **409**:2, 238-249. [CrossRef]
15. Matthias J Feige, Linda M Hendershot. 2011. Disulfide bonds in ER protein folding and homeostasis. *Current Opinion in Cell Biology* **23**:2, 167-175. [CrossRef]
16. Lei Wang, Li Zhu, Chih#chen Wang. 2011. The endoplasmic reticulum sulfhydryl oxidase Ero1# drives efficient oxidative protein folding with loose regulation. *Biochemical Journal* **434**:1, 113-121. [CrossRef]
17. Van Dat Nguyen, Mirva J. Saaranen, Anna-Riikka Karala, Anna-Kaisa Lappi, Lei Wang, Irina B. Raykhel, Heli I. Alanen, Kirsi E.H. Salo, Chih-chen Wang, Lloyd W. Ruddock. 2011. Two Endoplasmic Reticulum PDI Peroxidases Increase the Efficiency of the Use of Peroxide during Disulfide Bond Formation. *Journal of Molecular Biology* **406**:3, 503-515. [CrossRef]
18. Éva Margittai, Roberto Sitia. 2011. Oxidative Protein Folding in the Secretory Pathway and Redox Signaling Across Compartments and Cells. *Traffic* **12**:1, 1-8. [CrossRef]
19. Timothy J Tavender, Jennifer J Springate, Neil J Bulleid. 2010. Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. *The EMBO Journal* **29**:24, 4185-4197. [CrossRef]
20. Ester Zito, Eduardo Pinho Melo, Yun Yang, Åsa Wahlander, Thomas A. Neubert, David Ron. 2010. Oxidative Protein Folding by an Endoplasmic Reticulum-Localized Peroxiredoxin. *Molecular Cell* **40**:5, 787-797. [CrossRef]
21. Milena Bertolotti , Sun Hee Yim , Jose M. Garcia-Manteiga , Silvia Masciarelli , Yoo-Jin Kim , Min-Hee Kang , Yoshihito Iuchi , Junichi Fujii , Roberta Vené , Anna Rubartelli , Sue Goo Rhee , Roberto Sitia . 2010. B- to Plasma-Cell Terminal

Differentiation Entails Oxidative Stress and Profound Reshaping of the Antioxidant Responses. *Antioxidants & Redox Signaling* **13**:8, 1133-1144. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]

22. Vamsi K. Kodali , Colin Thorpe . 2010. Oxidative Protein Folding and the Quiescin–Sulfhydryl Oxidase Family of Flavoproteins. *Antioxidants & Redox Signaling* **13**:8, 1217-1230. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
23. Kenji Inaba, Shoji Masui, Hiroka Iida, Stefano Vavassori, Roberto Sitia, Mamoru Suzuki. 2010. Crystal structures of human Ero1# reveal the mechanisms of regulated and targeted oxidation of PDI. *The EMBO Journal* **29**:19, 3330-3343. [CrossRef]
24. Christian Appenzeller-Herzog, Jan Riemer, Ester Zito, King-Tung Chin, David Ron, Martin Spiess, Lars Ellgaard. 2010. Disulphide production by Ero1#–PDI relay is rapid and effectively regulated. *The EMBO Journal* **29**:19, 3318-3329. [CrossRef]
25. Éva Margittai, Gábor Bánhegyi. 2010. Oxidative folding in the endoplasmic reticulum: Towards a multiple oxidant hypothesis?. *FEBS Letters* **584**:14, 2995-2998. [CrossRef]
26. Miklós Csala , Éva Margittai , Gábor Bánhegyi . 2010. Redox Control of Endoplasmic Reticulum Function. *Antioxidants & Redox Signaling* **13**:1, 77-108. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
27. Anna-Riikka Karala, Lloyd W. Ruddock. 2010. Bacitracin is not a specific inhibitor of protein disulfide isomerase. *FEBS Journal* **277**:11, 2454-2462. [CrossRef]
28. Anna-Riikka Karala, Anna-Kaisa Lappi, Lloyd W. Ruddock. 2010. Modulation of an Active-Site Cysteine pKa Allows PDI to Act as a Catalyst of both Disulfide Bond Formation and Isomerization. *Journal of Molecular Biology* **396**:4, 883-892. [CrossRef]
29. Mirva J. Saaranen , Anna-Riikka Karala , Anna-Kaisa Lappi , Lloyd W. Ruddock . 2010. The Role of Dehydroascorbate in Disulfide Bond Formation. *Antioxidants & Redox Signaling* **12**:1, 15-25. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
30. Adam M. Benham. 2009. Protein folding and disulfide bond formation in the eukaryotic cell. *FEBS Journal* **276**:23, 6905-6911. [CrossRef]
31. Feras Hatahet , Lloyd W. Ruddock . 2009. Protein Disulfide Isomerase: A Critical Evaluation of Its Function in Disulfide Bond Formation. *Antioxidants & Redox Signaling* **11**:11, 2807-2850. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]