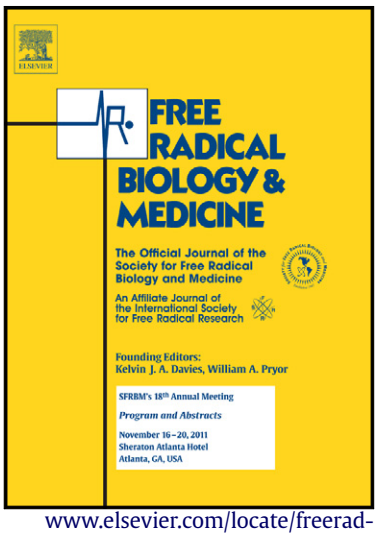

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Composition of the redox environment of the endoplasmic reticulum and sources of hydrogen peroxide

Éva Margittai[1], Balázs Enyedi[2], Miklós Csala[3], Miklós Geiszt[2,4], Gábor Bánhegyi[3*]

[1]Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary;

[2]Department of Physiology, Semmelweis University, Budapest , Hungary;

[3]Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary;

[4]"Lendület" Peroxidase Enzyme Research Group of the Semmelweis University and the Hungarian Academy of Sciences.

[*]Corresponding author:

G. Bánhegyi, Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, PO Box 260, Budapest 1444, Hungary. Phone: +36 1 4591500. E-mail: banhegyi@eok.sote.hu

Abbreviations: AA, ascorbic acid; DHA, dehydroascorbic acid; ER, endoplasmic reticulum; ERAD, ER-associated degradation; Gpx, glutathione peroxidase; GR, glutathione reductase; H6PD, hexose-6-phosphate dehydrogenase; Nox, NADPH oxidase; PDI, protein disulfide isomerase; Prx, peroxiredoxin; ROS, reactive oxygen species; VKOR, vitamin K epoxide reductase; 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1.

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ABSTRACT

The endoplasmic reticulum (ER) is a metabolically active organelle, which has a central role in proteostasis by translating, modifying, folding and occasionally degrading secretory and membrane proteins. The lumen of the ER represents a separate compartment of the eukaryotic cell, with a characteristic proteome and metabolome. Although the redox metabolome and proteome of the compartment have not been holistically explored, it is evident that proper redox conditions are necessary for the functioning of many luminal pathways. These redox conditions are defined by local oxidoreductases and the membrane transport of electron donors and acceptors. The main electron carriers of the compartment are identical with those of the other organelles: glutathione, pyridine and flavin nucleotides, ascorbate and others. However, their composition, concentration, and redox state in the ER lumen can be different from those observed in other compartments. The terminal oxidases of oxidative protein folding generate and maintain an "oxidative environment" by oxidizing protein thiols and producing hydrogen peroxide. ER-specific mechanisms re-utilize hydrogen peroxide as an electron acceptor of oxidative folding. These mechanisms, together with membrane and kinetic barriers, guarantee that redox systems in reduced or oxidized state can be present simultaneously in the lumen. The present knowledge is rather limited on the *in vivo* conditions of ER redox; development of new genetically encoded targetable sensors for the measurement of luminal state of redox systems other than thiol/disulfide will contribute to a better understanding of ER redox homeostasis.

Keywords: endoplasmic reticulum; redox; hydrogen peroxide; glutathione; NADPH; ascorbate; NADPH oxidases.

Introduction

The redox environment of a subcellular compartment is determined by the concentration and redox state of the major redox-active compounds (*i.e*. electron carriers composed by electron donors and acceptors). The redox conditions in the ER lumen got into the focus of interest by the recognition of oxidative folding and its major oxidoreductases [for recent reviews see 1-3]. It has been recognized soon that the thiol/disulfide redox system is present in an oxidized state comparing to the cytosol [4]. Due to the application of genetically encoded ER-targetable fluorescent sensors, the *in vivo* real time investigation the thiol/disulfide redox system became feasible. However, the knowledge on the other luminal redox systems [5,6] is regrettably scanty. Although indirect proofs show the presence of pyridine and flavin nucleotides, ascorbate and other redox-active compounds, their source, luminal concentration and redox state have not been defined. Thus, our present viewpont on ER redox has an oxidative folding-dependent focus. Indeed, the redox conditions in the ER seems to be organized according to the requirements of the oxidative protein folding:

- The main low molecular weight electron carriers are not synthesized in this compartment; they are taken up by – molecularly mostly undefined – transporters, creating a point for the regulation of luminal redox.
- The thiol/disulfide redox system is separated from the cytosolic one by a membrane barrier and from the pyridine nucleotide system by the absence of the adequate reductases. Thus, the luminal thiol/disulfide redox system can keep its oxidized state crucial for oxidative folding.
- Despite the intense oxidative activity, the ER lumen is not equipped with the common antioxidant enzymes; instead, special luminal enzymes utilize protein thiols as electron donors and reactive oxygen species (ROS) as electron acceptors. These conditions also promote the oxidative folding.

Disturbance of the luminal redox homeostasis in the ER is an almost universal consequence of various human pathologies affecting the organelle [7,8]. Redox imbalance by itself and by altering key ER functions is perceived as the ER stress [9], which activates various adaptive signaling pathways. Among these cellular responses, the unfolded protein response (UPR) is the best known, in accordance with the prominent role of the ER in the synthesis and folding of secretory proteins [10-12].

However, oxidative protein folding is not the only ER function that employs and affects luminal redox systems. The aim of the present review is to delineate the major redox systems of the ER and their connections and disconnections from a broader aspect including some points of view beyond oxidative folding. The main luminal hydrogen peroxide producing reactions and their impact on the local redox conditions as well as some experimental tools allowing their investigation are also covered.

Glutathione and its disulfide, the main redox buffer in the ER lumen

The major redox buffer of the ER lumen, similarly to other subcellular compartments, is composed of the glutathione/glutathione disulfide (GSH/GSSG) couple. However, the luminal GSH-GSSG pool has been found to be different from the cytosolic one regardig both the total concentration and the ratio of its two components. A pioneering study revealed a GSH/GSSG ratio of 1:1–3:1 in the ER and in the whole secretory pathway in hybridoma cells by using a small thiol-containing glycopeptide, which can undergo thiol-disulfide exchange with glutathione [4]. The calculated redox potential values were between −170 and −185 mV in the ER lumen in contrast to an approximately −230 mV in the cytosol. Similar GSH/GSSG ratios (3:1–6:1) were observed by using ER-derived microsomal vesicles [13,14].
Applications of genetically encoded targetable redox sensors have recently resulted in slightly different values. −208±4 mV GSH/GSSG redox potential was measured by using a glutathione-specific redox-sensitive variant of green fluorescent protein in the ER lumen of intact HeLa cells [15]. The authors concluded that the GSH/GSSG ratio was equal or above 11:1, or the total luminal concentration of glutathione exceeded the overall cellular concentration. The results were also confirmed in a recent study of the same group, by using an ER-targeted single-cysteine glutaredoxin, which rapidly attains equilibrium with GSH/GSSG through autocatalytic glutathionylation. The calculated GSH/GSSG ratio in the ER lumen of HeLa cells was below 7:1 and the luminal concentration of total glutathione was about twofold higher than the overall cellular concentration [16]. The cytosolic GSH/GSSG ratio is about three orders of magnitude higher than the ER luminal one in cultured human cells and reflects to a redox potential between –280 and –320 mV [17]. Therefore, it can be concluded that the composition of the ER luminal glutathione redox buffer is robustly shifted towards the oxidized/oxidizing direction as compared to the cytosolic conditions, and glutathione (mainly GSSG) accumulates in the ER lumen.
Since the enzymes of glutathione synthesis are absent from the ER, the luminal glutathione pool must be derived from the cytosol. The preferential uptake of GSSG was suggested to be responsible for the peculiar intraluminal milieu and to provide the oxidizing power for disulfide bond formation in secretory proteins [4]. However, considering a gradient across the ER membrane, GSSG import would imply an active pumping activity. Systematic analysis of microsomal glutathione traffic revealed that GSH rather than GSSG is preferentially transported across the ER membrane by facilitated diffusion [18]. Although such a transport is theoretically bidirectional, the physiological GSH concentrations favor the uptake of GSH

into the ER lumen. In addition to these contradictory findings, the theory of GSSG import was further weakened by the discovery of endoplasmic reticulum oxidoreductin (Ero) enzymes, which catalyze the final step of the electron transfer chain, which drives protein disulfide bond formation in the ER lumen [19,20].

GSH entering the ER lumen can be utilized in various local reactions, among which protein processing involves the most powerful oxidizing machinery. GSH is connected to the electron transfer chain of oxidative protein folding at multiple levels. Thiol/disulfide exchange can occur with substrate proteins, or various oxidoreductases including the initiator oxidases of the process. Thus, it was supposed that glutathione and protein thiols compete for the oxidizing power, which drives disulfide bond formation [21]. GSH acts as an electron donor for PDI; an *in vitro* study showed a rapid GSH-dependentreduction of the isolated *a* domain in either the human or the yeast enzyme protein [22]. Spontaneous, uncontrolled thiol/disulfide exchange between GSH and protein disulfides would remarkably worsen the efficiency of oxidative folding. Nevertheless, the GSH-dependent protein disulfide reductions are hampered by both membrane and kinetic barriers and are restricted to carefully balanced enzymatic activities. Thus, oxidoreductases involved in the oxidative protein folding depend on the redox state of their partner enzymes rather than on the actual state of the luminal redox buffer [23].

Members of the PDI family can reduce and isomerize inappropriate protein disulfides by using GSH as the source of electrons [24,25]. Similarly, GSH is also utilized in the reductive unfolding of irremediably misfolded proteins directed to the ER-associated protein degradation (ERAD). Since even a partial folding prevents these proteins from being retrotranslocated into the cytosol, the place of proteasomal degradation, all disulfides in the ERAD substrates must be first reduced [26]. Oxidoreductases catalyzing these reactions (*e.g.* ERdJ5) utilize GSH as electron donor [27]. It should be noted that ERdJ5 disulfide reductase is also involved in the isomerization of non-native disulfides as it was revealed in a study on the folding process of the LDL receptor [28].

Ero1, the terminal oxidase of oxidative folding, can also catalyze GSH oxidation. Indeed, the rate of GSH oxidation was shown to correlate with Ero1p activity in yeast: regeneration of GSSG after dithiothreitol treatment was delayed by Ero1p deficiency and accelerated by Ero1p overexpression [21]. However, *in vitro* experiments demonstrated that GSH is a poor substrate for Ero1 [29], suggesting that Ero1 activity promotes GSH oxidation indirectly through PDI or hydrogen peroxide.

One of the major GSH utilizing pathways of the cell is the elimination of hydrogen peroxide by glutathione peroxidases (GPx). This enzyme family is represented by GPx7 and 8 in the ER lumen, which have been proved to be effective PDI peroxidases (*i.e.* they can use reduced PDI instead of GSH as electron donor) [30-32]. However, at least in case of Gpx7, GSH can be an alternative substrate in the reaction, with a relatively low rate [33].

Due to the poor permeability of the ER membrane to GSSG [18], the intense local GSH oxidation in the aforementioned reactions results in the luminal accumulation of GSSG, which can still leave the compartment *via* the secretory pathway. GSSG can also be engaged in thiol-disulfide exchange reactions with substrate proteins of oxidative folding, although these reactions are kinetically unfavorable. *In vitro* experiments showed that supplementation with an appropriate glutathione redox buffer facilitated the folding of proteins, even in the absence of oxidoreductases [34]. However, addition of oxidoreductases such as PDI dramatically increased the rate of the reaction [35]. Thus, the relative contribution of the non-enzymatic reactions to the total performance of oxidative folding is presumably negligible in the luminal compartment of the ER. GSSG-dependent oxidation of human PDI and yeast Pdi1p was observed in vitro [22], and this phenomenon has been also demonstrated in Ero1-deficient cells as a possible alternative pathway for disulfide bond generation [36]. These findings suggest that the fine tuning of luminal redox homeostasis involves a direct thiol/disulfide exchange between GSH/GSSG and PDI, which also recycles the oxidizing power of GSSG into the machinery of oxidative folding [37].

The oxidizing power of GSSG is rarely utilized in the other subcellular compartments. Instead, GSH is regenerated by glutathione reductase (GR) at the expense of NADPH. The presence of GR in the ER lumen would tend to equalize the redox states of the two major redox systems in the ER lumen by oxidizing NADPH and reducing GSSG. However, this would simultaneously compromise both the functioning of luminal NADPH-dependent reductase enzymes (see later) and the oxidative protein folding. The absence of luminal GR activity ensures the uncoupling of GSG/GSSG and NADPH/$NADP^+$ redox systems [38]. Thus, oxidative protein folding is protected from reducing effects by dual mechanisms: cytosolic thiols and luminal disulfides are separated by a membrane barrier, while the colocalized disulfides and NADPH are isolated by a metabolic barrier.

The maintenance of the redox potential of the luminal GSH/GSSG buffer is required for appropriate protein folding. Thus, scrupulous regulatory mechanisms guarantee the constancy. In case of hyperreducing conditions (*e.g.* upon the addition of dithiothreitol), the activity of Ero isozymes quickly restore the normal redox state. In case of hyperoxidizing

conditions, at least two main compensatory mechanisms get activated. First, the activity of Ero1α is inhibited because the enzyme contains regulatory intramolecular disulfides, which can be reduced to activate and reoxidized to inhibit the enzyme [39,40]. The presence of reduced PDI keeps Ero1α in the active form, by reducing these regulatory disulfide bonds [39,40]. Interestingly, the inhibitory reoxidation catalyzed by specific members of the PDI family, PDI or ERp46, is not dependent on the concentration of molecular oxygen or hydrogen peroxide [41]. This mechanism guarantees that the electron transfer of oxidative protein folding is controlled by the availability of electron donors rather than electron acceptors (Figure 1).

The second possibility to cope with hyperoxidation is the intake of reducing compounds or the release of oxidants into/from the ER lumen. The uptake of GSH and/or the release of GSSG are the most evident solutions. Indeed, several observations indicate the presence of a luminal reductive shift during ER stress caused by various stressors, typically by calcium depletion [42-44]. The role of GSH in the phenomenon is supported by the finding that components of the oxidative folding pathway become hyperoxidized in the absence of cytosolic GSH in plasma membrane permeabilized cells, whereas the addition of GSH restores normal disulfide formation [25]. Thus, the presence of the cytosolic GSH pool and the transport of GSH are indispensable for proper oxidative folding. However, it is not clear whether the observed reductive shift is due to activation of the putative transporter responsible for basal GSH uptake or to contribution of alternative transport activities. The observation that luminal hyperoxidation through an Ero1-independent local hydrogen peroxide generation increases the general permeability of the ER membrane seems to support the latter option [45,46].

Calcium fluxes also play an important role in the regulation of luminal redox (and *vice versa*). Beside the above-mentioned phenomenon [42-44], luminal redox changes greatly affect both ER calcium ion channels and calcium ion pumps. ERp44, an ER resident member of the PDI protein family inhibits inositol 1,4,5-trisphosphate-induced calcium release via IP3R1. ERp44 directly interacts with the luminal region of IP3R1 in a pH, [$Ca2^{+}$] and redox state dependent manner [47]. The presence of reduced cysteinyl thiols is required for the interaction. Overexpression of Ero1α increases passive calcium efflux from the ER by regulating the interaction. The mitochondria-associated membrane (MAM) has been shown to be enriched in the components of this redox-dependent regulatory mechanism [48].

Calcium ion reuptake into the ER is also regulated by luminal redox state and calcium ion concentrations. ERp57 was shown to regulate sarco/endoplasmic reticulum calcium ATPase type 2b (SERCA2b) activity. ERp57 overexpression reduces the frequency of SERCA2b-dependent calcium oscillations, which was dependent on the presence of cysteinyl residues located in an intraluminal loop. Store depletion causes the dissociation of ERp57 and SERCA2b, and hence the termination of inhibition [49]. Thus, a feedback loop of regulation exists in ER redox and calcium handling: hyperoxidizing conditions within the ER result in the stimulation of calcium release, and the concomitant depletion of the calcium store promotes a luminal reductive shift (Figure 1).

Pyridine nucleotides in the ER

Dehydrogenases, reductases and monooxygenases of the metabolism often use NAD(H) and its phosphorylated derivative, NADP(H) (collectively termed as pyridine nucleotides) as water-soluble electron carriers (see [50] and references therein). These enzymes and their cofactors are presumably present in all subcellular compartments of the eukaryotic cell, including the ER lumen. However, reactions of pyridine nucleotide biosynthesis are localized to the cytosol and the mitochondria only [51]; the origin of the pyridine nucleotide pool in other compartments, such as the peroxisomes and the components of secretory pathway, is still elusive. Membranes are non-permeable towards pyridine nucleotides and transporters for pyridine nucleotides or for their proximate precursors have not been described. Anyway, the occurrence of some pyridine nucleotide-dependent enzymes has been reported in the ER lumen, which necessitates the presence of cofactors. Since the separate detection of NAD^+/NADH or $NADP^+$/NADPH *in vivo* or in cellular systems has not been possible, the available sparse data have been mainly collected in isolated microsomes with enzymatic methods [52,53]. Thus, the results not necessarily mirror the *in vivo* situation with respect to the composition, the concentration, or the redox state of the luminal pyridine nucleotides. A recently published method holds out a promise to solve these questions. By using targeted mutant isocitrate dehydrogenase enzymes and ^2H stable isotopes NAD(P)H metabolism was selectively observed in the cytosol and mitochondria, on the basis of 2-hydroxyglutarate formation [54].

Redox status of the pyridine nucleotide pool of the ER is tuned by luminal enzymes (Figure 2). NADPH is generated and maintained by luminal $NADP^+$-dependent dehydrogenases,

hexose-6-phosphate dehydrogenase (H6PD) being the most important among them [55,56]. Reducing equivalents arrive to the enzyme in form of glucose-6-phosphate *via* the glucose-6-phosphate transporter [57] of the ER. H6PD catalyzes the first two steps of the pentose phosphate pathway, thus the product of the reaction is 6-phosphogluconate. H6PD is characterized by low K_M toward glucose-6-phosphate and the absence of NADPH-dependent feedback inhibition. These features guarantee that H6PD can generate a very high [NADPH]/[$NADP^+$] ratio ([58] and references therein). Although the presence of other $NADP^+$-dependent dehydrogenases – *e.g.* isocitrate dehydrogenase [59] and malic enzyme [60] - has been observed, their contribution to the redox homeostasis of the ER lumen has not been clarified.

Among the luminal NADPH consuming enzymes 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) is the best known [61]. 11βHSD1 reversibly converts 11- and 7-oxosterols into the respective 7-hydroxylated forms; its activity is one of the major determinants of the local glucocorticoid hormone levels [62]. The direction of the reaction is largely dependent on the redox state of the NADPH/$NADP^+$ system [63]. In fact, the physiological direction of the process – reduction – is the strongest indirect evidence for a high ER luminal [NADPH]/[$NADP^+$] ratio, which has not yet been directly determined.

Other NADPH consuming enzymes may be also present in the ER lumen, such as ER flavoprotein associated with degradation (ERFAD), a putative component of ER-associated degradation (ERAD) [64], some members of the short-chain dehydrogenase/reductase family [65] and certain cytochrome P450 isozymes (*e.g.* CYP2W1) [66].

Luminal NAD^+ or NADH dependent reactions have been hardly explored. An interesting exemption is the presence of mono-ADP ribosyltransferases (*e.g.* ARTC1) in the compartment [67], which use Grp78 as substrate. Activation of mono-ADP ribosylation in nutritional stress [68], in ischemia-reperfusion [69] or in ER stress [70] diminishes the active pool of Grp78 and intensifies ER stress signaling. Luminal NAD^+ is also depleted in these conditions; it can be hypothesized that it might have an impact on the total pyridine nucleotide pool of the ER lumen (Figure 2).

Connections between pyridine nucleotides and other redox systems seem to be cut off in the ER lumen. While the NADPH/$NADP^+$ redox pair is tightly coupled to the GSH/GSSG and ascorbate (AA)/DHA systems by GR and DHA reductases [71-73] in the cytosol, these enzymes are absent or at least has not been observed in the ER [38]. The separation of the

redox systems is further supported by the following observations: the addition of GSH or GSSG does not influence the redox state of pyridine nucleotides in liver microsomes [38], and reduced or oxidized pyridine nucleotides are unable to affect the redox state of microsomal thiols [38] or influence oxidative protein folding [74].

Nevertheless, some observations suggest that indirect connections might exist between the two redox systems, including the possible competition between NADPH and thiols for hydrogen peroxide reduction and the putative involvement of NADPH in disulfide bond reduction during the ERAD [ERFAD paper]; however, these possibilities are waiting for experimental verification.

Due to the aforementioned technical difficulties, moderate insight has been gained concerning the regulation of the redox state of luminal pyridine nucleotides. Under physiological conditions the dietary supply of reducing equivalents (*i.e.* carbohydrates and lipids) maintains high NADPH/$NADP^+$ ratio, as it was also evidenced in cellular systems [63]. Restriction of nutrient supply in starvation resulted in decreased NADPH level and NADPH/$NADP^+$ ratio, measured in microsomes from rat liver [53]. The luminal NADPH/$NADP^+$ ratio can initiate signaling processes leading to death-or-survival decisions. NADPH depletion induces mTOR-dependent autophagy [75-78]. Thus, the concentration and redox status of luminal pyridine nucleotides might have an impact at cellular level, connecting the metabolism with cell fate decisions [79].

Other components of the luminal redox environment

Ascorbate

The AA/DHA redox couple is also present in the ER lumen (Figure 3). The source of luminal AA/DHA in the majority of vertebrate cells is a transmembrane transport from the cytosol, mediated by transporters less characterized at molecular level [80]. DHA seems to be the preferentially transported form; it was reported that in ER-derived microsomal vesicles DHA was taken up with the mediation of GLUT-type transporter(s) [81]. AA transport was hardly detectable, and it could be stimulated by oxidative effects [81,82]. GLUT10 has been recently proposed as an ER DHA transporter [83], but the fact that pathological alterations in its inherited deficiency (arterial tortuosity syndrome, [84]) is restricted to certain cell types only suggests that other ER DHA transporters must exist. It should be noted that partial

colocalization of the sodium-dependent ascorbate transporter SVCT2 and PDI, and the copurification of SVCT2 with the ER chaperon calnexin were also reported [85]. However, functional characterization of ascorbic acid transport in the ER is required to estimate its contribution to vitamin C uptake by the organelle.

DHA transport in the ER shows the characteristics of facilitated diffusion [81]. The physiological direction of DHA flux is unknown; it can be supposed that the oxidative luminal environment facilitates DHA formation, while the cytosolic reductive milieu promotes DHA reduction. These considerations can lead the assumption that there is a DHA gradient across the ER membrane, which would facilitate a lumen-to-cytosol diffusion. However, this way the ER ascorbate pool would be emptied. However, an AA oxidase activity associated with the outer surface of the ER membrane has been reported [86], which can revert the direction of the transport.

It should be noted that in vertebrates able to synthesize AA the last enzyme of the biosynthetic pathway, L-gulonolactone oxidase, is an ER-resident integral membrane protein of the liver or kidney, with an active site facing to the lumen. Ascorbate and equimolar hydrogen peroxide as byproduct [87] are formed directly in the ER lumen; thus, these cells do not rely on uptake mechanisms.

AA, beside its general antioxidant role, is utilized as a cofactor for luminal Fe(II)/2-oxoglutarate-dependent dioxygenases: prolyl- and lysyl-hydroxylases (Figure 3). AA is required for the maintenance the redox state of ferrous iron at the active site of the enzymes [88]. Vitamin C behaves as an electron donor in these reactions. However, its electron acceptor function might also be present in the ER. DHA, either formed in local reactions or transported into the lumen of the ER can be reduced by dithiols in unfolded or partially folded proteins in a PDI-independent manner [89] or directly by PDI, oxidizing the active central dithiols of the enzyme; oxidized PDI reacts with reduced substrate proteins yielding protein disulfides and catalytically regenerating PDI [90].

In vivo findings also prove that luminal electron donor and electron acceptor functions of vitamin C mutually supports each other. Observations in scorbutic guinea pigs showed that not only prolyl- and lysyl-hydroxylations were defective, but ER stress also developed, presumably due to the missing prooxidant effect and the consequent impairment of oxidative protein folding [91]. Moreover, compromising the oxidative protein folding in mice with combined loss-of-function mutations in genes encoding the ER thiol oxidases ERO1α and

ERO1β, and peroxiredoxin 4 resulted in minor alterations only in disulfide bond formation, suggesting the existence of alternative electron transfer pathways. However, decreased hydroxyproline formation and defective intracellular maturation of procollagen were observed. Ascorbate depletion was also present, due to the consumption of ascorbate as a reductant for sulfenylated protein intermediates of oxidative folding, which were formed in the absence of ERO1α, ERO1β, and peroxiredoxin 4 [92,93]. The competition between protein sulfenic groups and prolyl hydroxylases for AA in the ER lumen ultimately resulted in impaired procollagen hydroxylation and in the alterations of extracellular matrix, *i.e.* in an unorthodox form of scurvy. These results clearly show that AA depletion can be limited to a compartment of the cell, *i.e.* ascorbate compartmentation diseases might exist [80]. Compartment-level detection of ascorbate concentrations and redox state has been unresolved problems. A method for organelle-specific detection of ascorbate has been recently published; the authors used immunocytohistochemistry combined with computer supported transmission electron microscopy [94,95]. The technique, which was applied in plant cells, allows the simultaneous investigation of changes in the subcellular distribution of ascorbate in the major compartments of the cell in a single experiment.

Flavin nucleotides

Thiol oxidases of the oxidative protein folding utilize tightly bound and free FAD for the reduction of oxygen [96,97]. FAD synthesized in the cytosol [98] can enter the ER lumen by a transporter unidentified at molecular level [99] and promotes Ero1-catalyzed oxidation. Addition of FAD facilitates luminal thiol oxidation both in yeast and liver microsomes, which suggest a regulatory role of FAD transport in oxidative folding.

Although the metabolism of FAD in the ER lumen has not been revealed, its redox-dependent regulation was observed in other compartments. A thiol-disulfide redox switch was reported in the human FAD synthase isoform 2. Accordingly, FAD synthesis was inhibited by thiol-blocking reagents, suggesting the involvement of free cysteines in the catalytic mechanism [100]. FAD degradation catalyzed by mitochondrial FAD pyrophosphatase was found to be a target of a NAD(H) dependent inhibition in *S. cerevisiae* [101]. The K*i* value of NAD^+ was one order of magnitude lower than that of NADH. A nuclear pool of FAD and FAD metabolizing enzymes was observed in rat liver; and the nuclear FAD pyrophosphatase could be also inhibited by adenylate-containing nucleotides [102]. Further research is needed to

explore the regulation of luminal FAD pool in the ER [103] and its physiological implications.

Vitamin K

γ-carboxylation, a posttranslational modification of vitamin K-dependent proteins occurs in the lumen of the ER. During the carboxylation reaction vitamin K is epoxidated, and ancillary reactions are needed for the regeneration of the cofactor. Vitamin K epoxide reductase (VKOR) contains a thioredoxin-like CXXC center involved in the reduction, which is linked to the oxidative folding of proteins in the ER by PDI. The formation of a stable complex between PDI and VKOR has also been suggested [104]. It can be concluded that electrons required for γ-carboxylation of proteins are provided by the cysteinyl thiols of substrate proteins of oxidative folding via PDI. The relative contribution of vitamin K pathway to oxidative folding was investigated in human hepatoma cells by using RNA interference. Combined depletion of Ero1 and peroxiredoxin 4 resulted in viable cells with maintained protein secretion, while the collective depletion of Ero1, peroxiredoxin 4 and VKOR activities resulted in cell death [105]. Thus, the PDI – VKOR – oxygen electron transfer chain contributes significantly to the oxidative folding.

Formation and elimination of hydrogen peroxide in the ER

The luminal compartment of the ER has emerged as a major site of intracellular reactive oxygen species, in particular hydrogen peroxide production (Figure 4). Hydrogen peroxide can be generated in these reactions as a byproduct or as a result of a "chemical accident". The majority of ER oxygenases and oxidases (*e.g.* cytochrome P450s, flavin-containing monooxygenases) are integral membrane proteins with their active sites facing the cytosol. However, many oxidoreductases are present in the ER lumen too; their activities greatly determine the luminal redox environment. The terminal oxidases of oxidative protein folding are the most powerful ones from this point of view. They are responsible for about one-fourth of the reactive oxygen species produced in a professional secretory cell [29]. Reduction of molecular oxygen by recombinant yeast Ero1p yields stoichiometric hydrogen peroxide under aerobic conditions [106]. A differentiated B lymphocyte produces about 10^5 disulfide bonds per second [107]; and hence the cellular hydrogen peroxide concentration would rise by 3 mM per hour without efficient mechanisms of elimination. Enhanced hydrogen peroxide

generation by the oxidative protein folding machinery has been proposed as a causative factor of ER stress triggered by the overproduction of secretory proteins [108].

Beside the terminal oxidases of oxidative protein folding, other luminal sources of hydrogen peroxide should be taken account. NADPH oxidase enzymes (Noxes) are among the less characterized sources of hydrogen peroxide in the ER. ROS production increases under conditions of ER stress, which has recently been attributed to the induction of Nox4 and Nox2, producing hydrogen peroxide and superoxide, respectively [109]. Members of the Nox/Duox family of NADPH oxidases are now considered as the primary, regulated sources of ROS [110-112]. The prototype Nox protein, Nox2 (formerly known as $gp91^{phox}$) was first identified in phagocytic cells as the key subunit of a multi-component enzyme complex, the phagocytic oxidase [113]. During the engulfment of invading pathogens the phagocytic oxidase transfers electrons from NADPH to molecular oxygen resulting in the formation of ROS, which contribute to microbial killing in the phagosome [113]. Other members of the mammalian Nox/Duox family of NADPH oxidases include Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2, respectively [111]. Discovery of the novel isoforms provided the long-sought molecular evidence for the regulated production of ROS in non-phagocytic cells and tissues. ROS production by non-phagocytic Noxes is essential for the physiological function of organs such as the thyroid gland and inner ear, however, the biological function of several Nox/Duox isoforms remains to be identified [112]. Although the ER has a well-established role in the synthesis of Noxes, recent data suggest that this organelle might also be an important location of Nox-derived hydrogen peroxide production [114]. Several studies located Nox4 to the ER, although the enzyme was also detected at other intracellular locations, including the nucleus, plasma membrane and mitochondria [114,115] It is important to note that the absence of specific antibodies often makes the results of Nox4 immunodetection experiments questionable, and hence the intracellular localization of Nox4 is a subject of debate [116]. Nevertheless, association of Nox4 to the ER seems highly likely, but the exact function of the enzyme in this organelle remains to be clarified. The potential targets of Nox4-derived hydrogen peroxide in the ER include protein-tyrosine phosphatase 1B [117,118], sarco/endoplasmic reticulum Ca^{2+}-ATPase [119] and ryanodine receptor [118]. These findings suggest a role for ER-localized Nox4 in growth factor receptor signaling and calcium handling. Since Nox4 is a constitutive generator of hydrogen peroxide, it is hard to envision its involvement in rapid signaling events, unless other, hitherto unknown mechanisms can acutely modulate its activity. Hydrogen peroxide production by Nox4 is

probably controlled at transcriptional level and an increase in Nox4 expression level was described during the unfolded protein response [109,120], where hydrogen peroxide production by Nox4 might constitute a proximal event of the response [121].

Recent findings indicate a role of Nox4 in the regulation of autophagy. Nutrient starvation (glucose deprivation) resulted in increased autophagy accompanied by elevated protein levels and hydrogen peroxide producing activity of Nox4 in cardiomyocytes [122]. Autophagy could be prevented by the selective knockdown of Nox4 or by overexpressing an ER-targeted catalase. Increased activity of the PERK branch of UPR was also observed. In line with these results, autophagy and Nox4 were activated during fasting and prolonged ischemia in the mouse heart *in vivo*, confirming that Nox4 is indispensable for activation of autophagy and cardioprotection [122,123]. It should be noted that beside increased ER hydrogen peroxide production, NADPH depletion also promotes autophagy (see above, refs [75-79]). Thus, increased Nox4 activity can act by a dual mechanism.

It is important to note that hydrogen peroxide produced by Nox4 is readily detectable in the extracellular space, thus the ER localization of Nox4 does not necessarily mean that all targets are confined to the same organelle. For example, Nox4 can regulate gene expression in endothelial cells, where Nox4-derived hydrogen peroxide increases the levels of endothelial nitric oxide synthase and heme oxygenase-1 [124,125].

Beside the above outlined sources of luminal hydrogen peroxide, the ER is presumably equipped with other oxidoreductases that can contribute to hydrogen peroxide production. However, the ER proteome and the topology of ER enzymes are poorly characterized [126], and to date, only the oxidases discussed here have been implicated as major determinants of ER redox conditions through luminal hydrogen peroxide production.

Despite the intense oxidative activity, the ER lumen is characterized by a surprisingly poor enzymatic antioxidant defense (Figure 4). The presence of superoxide dismutase, catalase and glutathione reductase has not been reported in the compartment. On the other hand, the lumen is equipped with special glutathione peroxidases (Gpx 7 and 8) and a peroxiredoxin (Prx4) [30,127,128]. These enzymes transfer electrons from glutathione [33], protein thiols [128] or PDI [33,127] to hydrogen peroxide. The source of thiol-oxidizing hydrogen peroxide can be the Ero1-catalyzed reaction [32,127]. However, it was also observed that Ero1-independent luminal hydrogen peroxide generation – *via* gulonolactone oxidase activity – could also stimulate disulfide bond formation [45]. Moreover, hydrogen peroxide itself was able to

promote disulfide bond formation, *i.e*. oxidation of thiol groups in GSH or in PDI. Hydrogen peroxide addition resulted in the rapid and efficient refolding of the model protein bovine pancreatic trypsin inhibitor [34].

In addition to the remarkable local formation, the luminal hydrogen peroxide status is also affected by export to and import from the cytosol. Hydrogen peroxide molecule is similar to water, thus it is not surprising that the water channel aquaporins can also facilitate the traffic of hydrogen peroxide across cellular membranes [129]. Specific aquaporins – peroxiporins – are especially engaged in hydrogen peroxide transport [129,130], and some representatives of these hydrogen peroxide-channels are present in the ER membrane. However, the actual direction of hydrogen peroxide flux has not been elucidated. Overexpression of a hyperactive Ero1α mutant resulted in ER stress, but not in a general antioxidant response of the cell [131]. This observation suggests that local hydrogen peroxide overproduction can be taken care of within the ER. Indeed, it was found that Gpx8, which is induced in ER stress, is able to prevent hydrogen peroxide leakage from the ER in this model [31]. Thus, the ER cannot be regarded as a net source of hydrogen peroxide even during stimulated protein folding.

Besides the antioxidant-dependent elimination of reactive oxygen species, recent observations suggest alternative mechanisms. *In vivo* generation of hydrogen peroxide in the ER lumen (by the stimulated activity of gulonolactone oxidase) was accompanied by transient liver swelling and reversible dilatation of the ER. Moreover, it resulted in an increased permeability of the microsomal membrane to various compounds of low molecular weight [46]. Thus, luminal hydrogen peroxide formation in the ER provokes a temporary increase in nonselective membrane permeability, which results in enhanced transmembrane fluxes of small molecules. Nevertheless, the increased permeability of the ER membrane would allow the escape of oxidizing agents from the lumen and the influx of reducing equivalents (*e.g.* GSH) from the cytosol.

Taken together, the oxidative strategy of the ER seems to be subordinated to the demands of oxidative protein folding (Figure 4). Reactions that could eliminate hydrogen peroxide through an unprofitable way are absent from the lumen; while hydrogen peroxide generated during disulfide bond formation are utilized for the production of further disulfide bonds. In fact, the two concommittant reactions deliver four thiol-derived electrons to molecular oxygen, which is finally reduced to water, and so the oxidative burden on the luminal environment and on the cell is economically evaded.

Connections between the redox systems of the ER – the role of hydrogen peroxide

The main electron carriers of the cell are integrated into a hierarchic network, referred to as Halliwell – Asada – Foyer cycle. However, in the ER lumen the electron flow is blocked between pyridine nucleotides and glutathione due to the absence of glutathione reductase. Thus, redox-active compounds of the lumen form a bi-central network around PDI and pyridine nucleotides as a result of uncoupling between the thiol/disulfide and the $NADPH/NADP^+$ systems [5]. The thiol/disulfide system intimately interacts with ascorbate, FAD and vitamin K; these (alternative) electron acceptors can promote oxidative protein folding. Less is known about the luminal connections of the pyridine nucleotide system. The uncoupling allows pyridine nucleotides to be present in a dominantly reduced form despite their co-localization with the characteristically oxidized thiol/disulfide system. The two systems, however, can theoretically communicate through a competition for ROS – mainly for hydrogen peroxide produced in association with oxidative folding. However, the priority of thiol oxidation in this putative competition seems to be ensured by multiple, efficient enzymatic and nonenzymatic reactions. The means of the electron transfer between thiols and hydrogen peroxide (glutathione peroxidases, peroxiredoxin 4) have been already characterized; further studies are needed to explore the corresponding reactions between pyridine nucleotides and hydrogen peroxide in the ER lumen.

Hydrogen peroxide sensors and their application in the ER

The two main approaches for measuring hydrogen peroxide in live cells are either using small molecular probes or genetically encoded fluorescent sensors. Oxidant sensitive dyes such as dihydrodichlorofluorescein are commonly used, however, they are generally not specific and selective, and react indirectly with hydrogen peroxide in most cases [132]. Although some of the cell-permeable dyes are enriched in certain organelles, such as the mitochondria, further chemical engineering would be required to target them to the ER.

Non-redox probes are currently an emerging family of dyes that sense ROS through a reaction, which, instead of oxidation, removes a protective group from the fluorophore. Boronate derivatives, the founding members of the group, were originally described as specific hydrogen peroxide probes [133]. Further experiments, however, proved their strong reactivity towards peroxynitrite and hypochlorous acid as well [134]. Although the SNAP-tag

technology allows ER-targeting of boronate probes [135], they react slowly and irreversibly with hydrogen peroxide. Thus, further development is required before they could be useful for measuring ER hydrogen peroxide levels.

Currently the best approach to measure hydrogen peroxide in the lumen of the ER is using fluorescent protein-based sensors. As a common feature, these probes incorporate a low pKa reactive cysteine, which is directly oxidized by hydrogen peroxide [136]. HyPer, the first genetically encoded hydrogen peroxide sensor was designed by inserting a circularly permuted yellow fluorescent protein (cpYFP) into the regulatory domain of OxyR, a bacterial hydrogen peroxide -sensing transcription factor [137]. The low pKa Cys199 of the regulatory domain lies deep within a hydrophobic pocket, which allows access exclusively to small, uncharged molecules such as hydrogen peroxide. Upon oxidation, Cys199 forms an intramolecular disulfide bond with Cys208, resulting in a dramatic conformational transition, which can be conveniently measured as a ratiometric signal change due to the fluorescent spectrum shift of cpYFP [138]. HyPer-3 is an optimized version of HyPer, with a twofold expanded dynamic range [139]. A long sought red version of the probe, HyPerRed has also been published recently [139]. Localization signals added to the probes can target them to various intracellular compartments, allowing site-specific hydrogen peroxide measurements [140]. A potential confounding factor in using HyPer is the strong pH-sensitivity, which, in alkalizing conditions, results in a fluorescence shift that resembles oxidation. This should be controlled for by verifying the results with the hydrogen peroxide-insensitive cysteine-mutant of HyPer (C121S), which retains its pH sensitivity without reacting to oxidation.

Several groups have found that ER-targeted HyPer is in an oxidized state [140-142], which could be attributed to high luminal hydrogen peroxide production. In the unique environment of the ER, HyPer may also reflect the activity of the disulfide-forming machinery, independent of hydrogen peroxide levels. In an attempt to support this, peroxiredoxin 4 overexpression was shown not to affect the resting HyPer-ER signal [142]. However, further experiments have demonstrated that GPx8 and not peroxiredoxin 4 seems to be the main detoxifying enzyme in the ER, which removes Ero1α-derived hydrogen peroxide [31]. Moreover, overexpression or depletion of GPx8 clearly affects HyPer-ER signals [31]. Despite these reaffirming results, HyPer measurements in the ER should be interpreted with caution, as they are likely to represent a combination of hydrogen peroxide production and the activity of the disulfide-forming machinery. Developing novel fluorescent probes or chemical dyes, which detect hydrogen peroxide independently of thiol oxidation reactions

and allow quantitative hydrogen peroxide measurements in the ER, would be a major contribution to the field.

Concluding remarks

The present state-of-art of ER redox homeostasis, due to the availability of appropriate experimental tools, is rather limited to the thiol-disulfide redox system and its relation to oxidative folding. The future development of ER-targeted redox sensors specific to pyridine nucleotides, ascorbate or hydrogen peroxide, as well as the mapping of the ER redox proteome are indispensable for the recognition of ER redox and understanding its connections with the cellular metabolic pathways. Further studies on ER redox-based signaling will definitely be resulted in important findings related to nutrient sensing, ER stress mechanisms and death-or-survival decisions. Taking together, research of ER redox will provide further insight into the pathomechanism of ER stress-related human diseases, such as diabetes, neurodegeneration, and cancer.

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Legend to Figures

Figure 1. Regulation of the thiol/disulfide redox state in the ER

In case of hypooxidizing conditions, excess thiols (including protein thiols, GSH or even xenobiotics) are rapidly oxidized by the electron transfer chain of oxidative protein folding, restoring the normal redox state. Hyperoxidizing conditions inhibit the electron transfer at the level of Ero1, and deplete the luminal calcium pool via redox control of calcium channels and pumps. In turn, calcium depletion opens/activates a yet-unidentified channel/transporter, which can rise the luminal thiol:disulfide ratio by allowing GSH influx, GSSG efflux, or both.

Figure 2. Pyridine nucleotides in the ER

ER pyridine nucleotides are taken up or synthesized locally by mechanisms presently unidentified. Luminal $NADP^+$ is reduced by H6PD to NADPH, which is reoxidized by 11βHSD1 and probably by other oxidoreductases. NAD^+ and NADH dependent enzymes are hardly known in the ER lumen. However, NAD^+ can be consumed by mono-ADP ribosylation reactions decreasing the luminal NAD^+ (and perhaps the total pyridine nucleotide) pool.

Figure 3. Ascorbate in the ER

Ascorbate (AA) produced by gulonolactone oxidase (GLO) in the ER of hepatocytes of ascorbate-synthesizing species or regenerated by the reduction of dehydroascorbic acid (DHA) supports the catalytic function of luminal Fe(II)/2OG dependent dioxygenases (prolyl- and lysyl-hydroxylases) and reduce hyperoxidized protein thiols. DHA, which is presumably transported by a GLUT-type transporter, can efficiently oxidize protein- and non-protein thiols in the ER lumen.

Figure 4. ROS handling in the ER

ROS production and ROS elimination are both connected to oxidative protein folding in the ER. Dark/thick arrows represent typical reactions, while pale/thin arrows represent minor

pathways of the compartment. Antioxidant enzymes absent or not reported in the ER lumen are shown on grey background. Abbreviations: AA, ascorbic acid; Apx, ascorbate peroxidase; DHA, dehydroascorbic acid; Gpx, glutathione peroxidase; Prx, peroxiredoxin.

HIGHLIGHTS

- Electron carriers imported to the ER establish isolated redox systems in the lumen
- The ER lumen is equipped with both dominantly reduced and oxidized redox couples
- Oxidant byproducts are utilized for and eliminated by disulfide formation in the ER

In vivo, real time detection of the main redox components in the ER is to be achieved

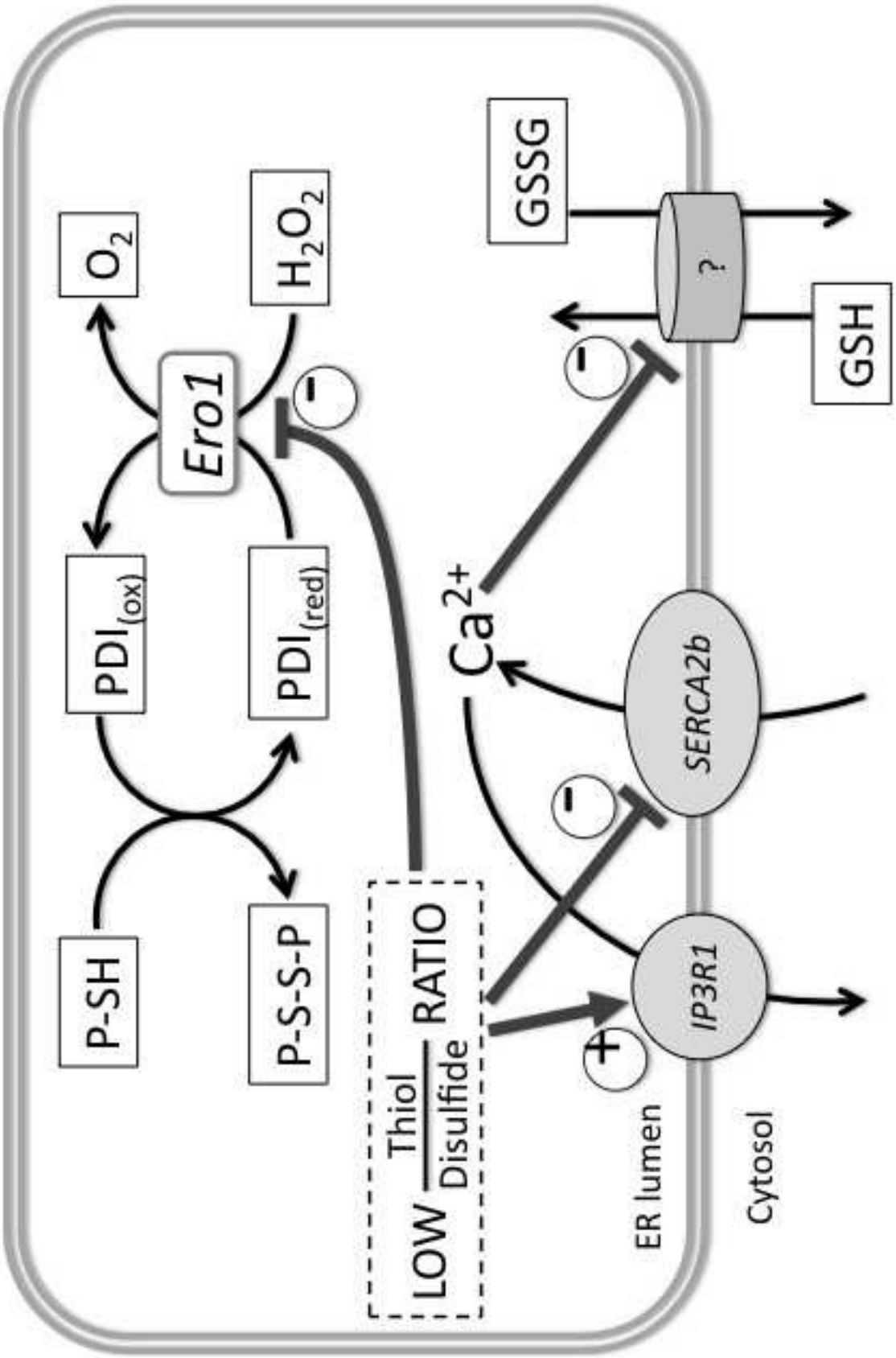

P-SH
P-S-S-P
PDI(ox)
PDI(red)
Ero1
O2
H2O2
Thiol
Disulfide
LOW
RATIO
Ca2+
IP3R1
SERCA2b
GSSG
GSH
?
ER lumen
Cytosol

Figure 1

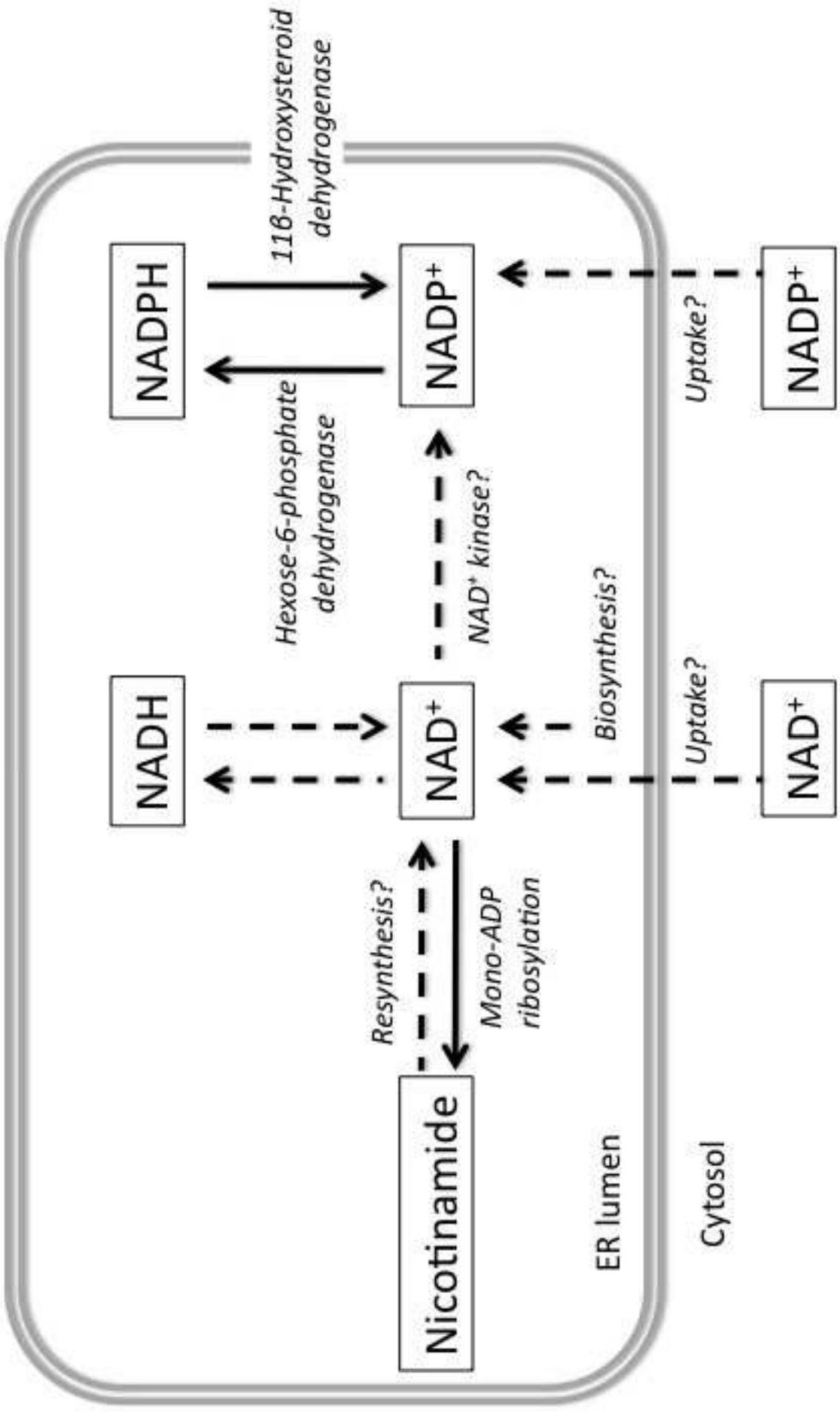
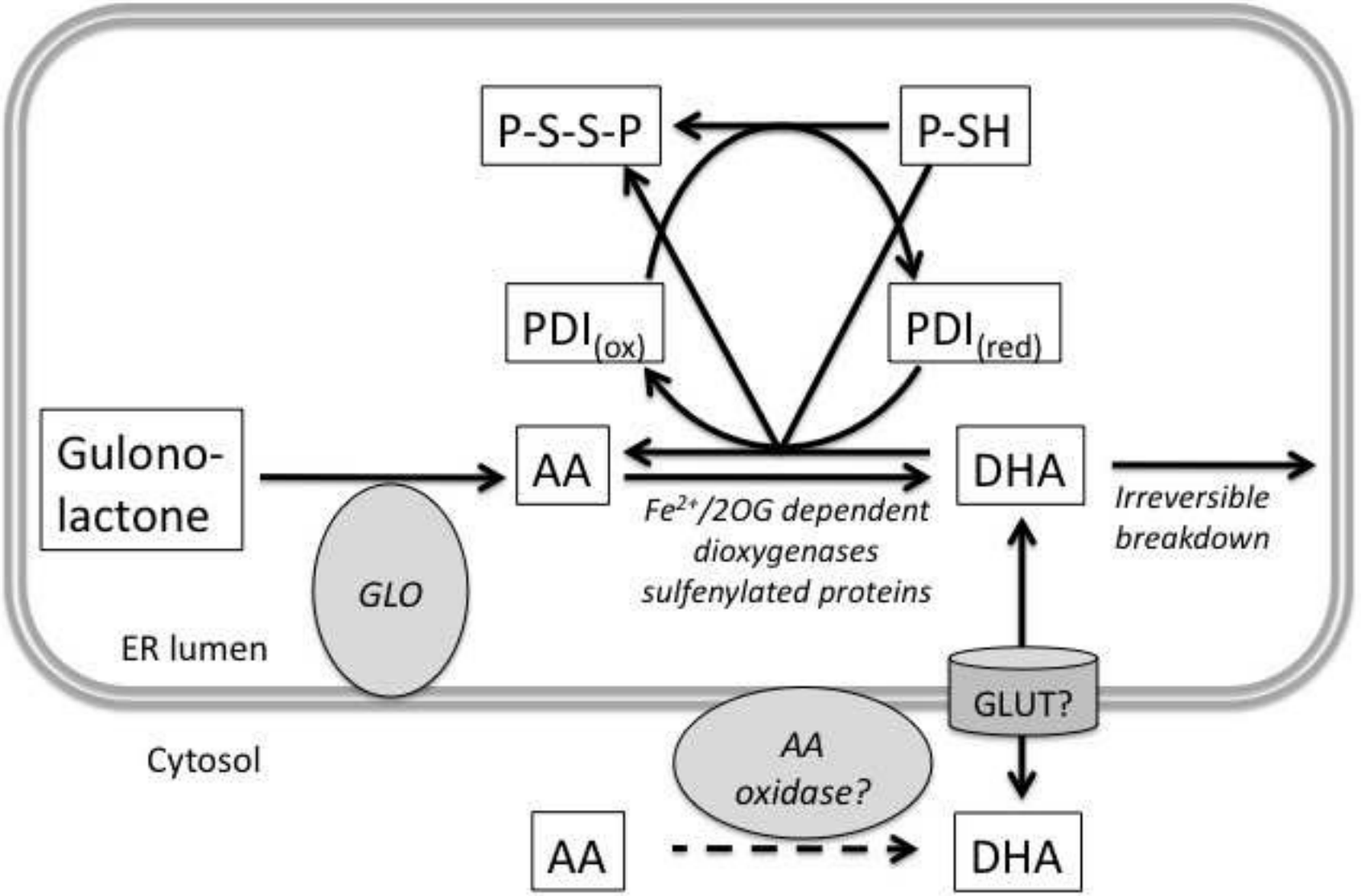

11β-Hydroxysteroid dehydrogenase
NADPH
NADP+
Hexose-6-phosphate dehydrogenase
NAD+ kinase?
Uptake?
NADP+
NADH
NAD+
Biosynthesis?
Uptake?
NAD+
Resynthesis?
Mono-ADP ribosylation
Nicotinamide
ER lumen
Cytosol

Figure 2

Figure 3



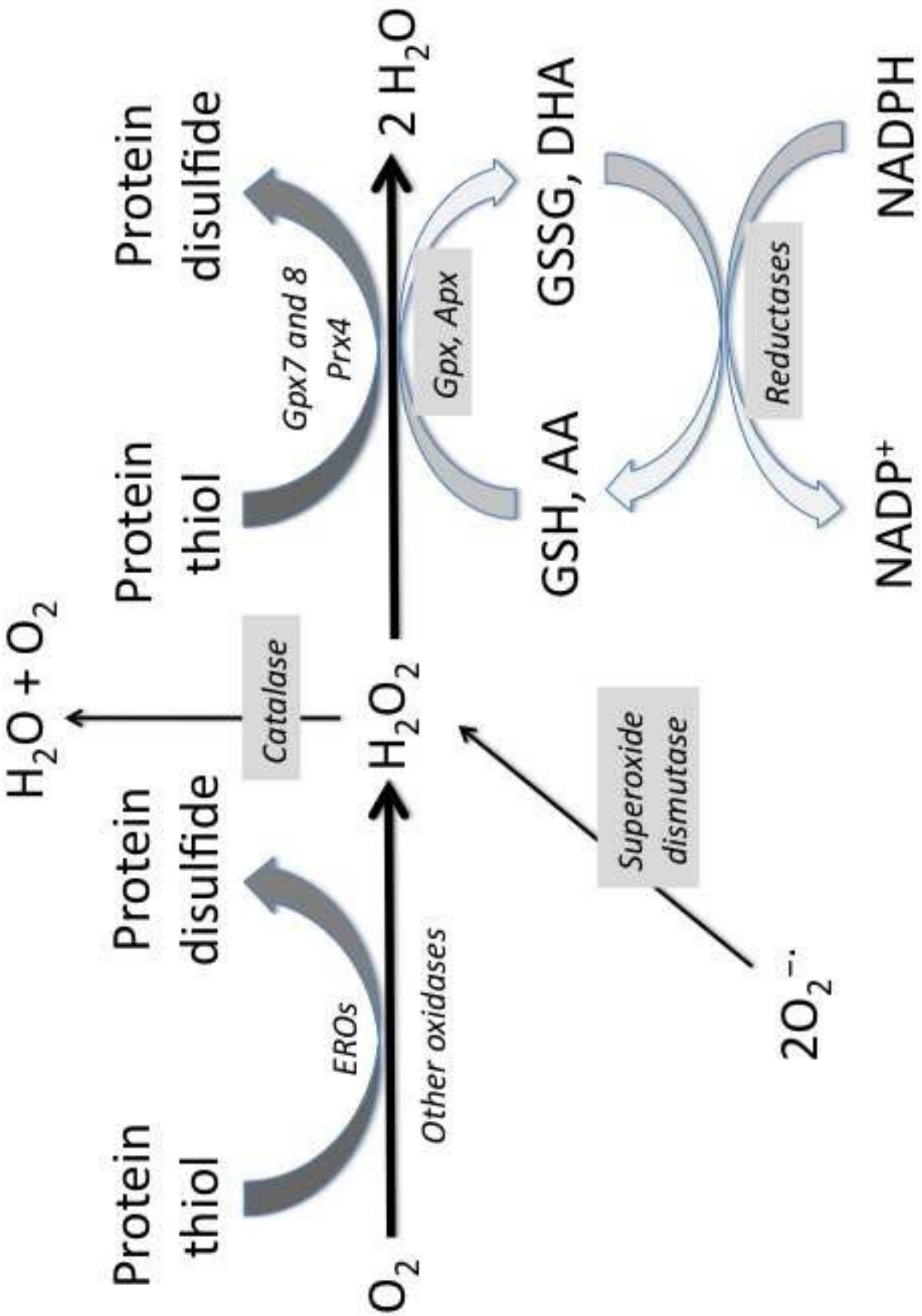


Figure 4