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REVIEW ARTICLE

The role of ascorbate in protein folding

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Abstract Ascorbate was linked to protein folding a long time ago. At the first level of this connection, it had been shown that ascorbate functions as an essential cofactor in the hydroxylation enzymes involved in collagen synthesis. Although the hydroxylation reactions catalyzed by the members of the prolyl 4-hydroxylase family are considered to be ascorbate dependent, the hydroxylation of proline alone does not need ascorbate. Prolyl 4-hydroxylases participate in two catalytic reactions: one in which proline residues are hydroxylated, while 2-oxoglutarate is decarboxylated and molecular oxygen is consumed. This reaction is ascorbate independent. However, in another reaction, prolyl 4-hydroxylases catalyze the decarboxylation of 2-oxoglutarate uncoupled from proline hydroxylation but still needing molecular oxygen. At this time, ferrous iron is oxidized and the protein is rendered catalytically inactive until reduced by ascorbate. At the second level of the connection, the oxidation and the oxidized form of ascorbate, dehydroascorbate, is involved in the formation of disulfide bonds of secretory proteins. The significance of the dehydroascorbate reductase activity of protein disulfide isomerase was debated because protein disulfide isomerase as a dehydroascorbate reductase was found to be too slow to be the major route for the reduction of dehydroascorbate (and formation of disulfides) in the endoplasmic reticulum lumen. However, very recently, low tissue ascorbate levels and a noncanonical scurvy were observed in endoplasmic reticulum thiol oxidase- and peroxiredoxin 4-compromised mice. This novel observation implies that ascorbate may be involved in oxidative protein folding and creates a link between the disulfide bond formation (oxidative protein folding) and hydroxylation.

Keywords Ascorbate · Dehydroascorbate · Ascorbate oxidase · Protein folding · Disulfide bond · Scurvy

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Introduction

During protein folding, proteins assume their functional conformation. Ascorbate was associated with protein folding a long time ago. The first level of this association originates from the time of the big biochemical discoveries and is connected to the elucidation of scurvy. Ascorbate functions as an essential cofactor in the hydroxylation of enzymes involved in collagen hydroxylation and as such underlies the molecular pathogenesis of the disease. The second level of the association of ascorbate and protein folding is connected to a very important basic biochemical observation. Together with the isolation of protein disulfide isomerase (PDI), a heat-stable factor was described in the formation of disulfide bonds (oxidative protein folding). This heat-stable factor could be replaced by the oxidized form of vitamin C, dehydroascorbate (DHA). Hence, dehydroascorbate became a candidate for the role of the electron acceptor for the electrons coming from reduced thiol-containing proteins via the sulfhydryl oxidase PDI. The significance of this electron acceptor role has recently been disputed. However, a noncanonical scurvy observed in endoplasmic oxidoreductin 1 (ERO1)α and ERO1β (the main sulfhydryl oxidases of the ER) and peroxiredoxin 4 (PRDX4) (ER-resident peroxiredoxin) triple mutant mice

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suggests an important role for ascorbate in oxidative folding and creates a link between disulfide bond formation (oxidative protein folding) and hydroxylation.

Ascorbate and protein hydroxylation

Protein hydroxylation is a posttranslational modification, involving the introduction of hydroxyl groups in the residues of substrate polypeptides which can function as further sites for glycosylation. The most notable example of protein hydroxylation is involved in collagen maturation. Incomplete collagen hydroxylation leads to the pathological condition known as scurvy, which is underpinned by the instability of collagen structure. It is well known that scurvy is prevented by sufficient ascorbate (vitamin C) consumption. It has also been shown that ascorbate functions as an essential cofactor for the hydroxylation enzymes involved in collagen synthesis and as such forms the basis for the molecular pathogenesis of the disease. The vital role of ascorbate encouraged further studies on hydroxylases and has led to the recognition of the 2-oxoglutarate-dependent dioxygenase family of proteins including prolyl 4-hydroxylase (P4H, EC 1.14.11.2) and lysyl 5-hydroxylase (L5H, EC 1.14.11.4), which have a diverse function and at least one common trait—the necessity for ascorbate.

Hydroxylation of collagen

The formation of a thermostable collagen structure at the temperature of the human body is associated with the hydroxylation of proline and lysine residues in the protein, resulting in 4-hydroxyproline (4-Hyp) and 5-hydroxylysine (5-Hyl). The extent and pattern of hydroxylation is dependent on age, tissue, and collagen type (Ruotsalainen et al. 2006). At the beginning of the investigations, it was theorized that proline and lysine hydroxylation is catalyzed by a single procollagen hydroxylase, because of the similar substrate and cofactor preference (Kivirikko and Prockop 1967) until further clarification (Kivirikko et al. 1972). Collagen forms a triple helical structure, and extensive posttranslational modifications have a major effect on its molecular assembly and stability.

Proline hydroxylation occurs at -X-Pro-Gly- triplets, and the reaction is catalyzed by collagen prolyl 4-hydroxylase (C-P4H), an $\alpha_2\beta_2$ heterotetramer, localized in the ER lumen, requiring 2-oxoglutarate, oxygen, ferrous iron, and ascorbate for its function (Fig. 1). While the α subunit participates directly in the hydroxylation, the β subunit of the complex was identified as the enzyme protein disulfide isomerase (PDI, EC 5.3.4.1), a member of the oxidative protein folding pathway in the ER lumen which functions to retain the assembly in this compartment (Myllylä et al. 1984; Kivirikko et al. 1989; Pihlajaniemi et al. 1991; Myllyharju 2003, 2008; Braakman and Bulleid 2011).

Lysine hydroxylation in collagen at X-Lys-Gly sites is catalyzed by lysyl 5-hydroxylase, which similar to P4H, has the cofactors 2-oxoglutarate, ferrous iron, and oxygen. L5H is also found in the ER lumen and forms a homodimeric complex (Turpeenniemi-Hujanen et al. 1980).

There are a number of pathological connective tissue disorders associated with lysine hydroxylase. Mutations in the gene coding L5H-1 isoenzyme (PLOD1) result in a rare pathological condition known as kyphoscoliosis (Ehlers–Danlos syndrome type VI (EDS VI)) featuring skin hyperextensibility, articular hypermobility, and tissue fragility. The major characteristics of EDS VI are severe muscle hypotonia at birth, generalized joint laxity, scoliosis at birth, and scleral fragility and rupture of the ocular globe (Beighton et al. 1998). Mutations in the isoenzyme L5H-2 (gene PLOD2) causes Bruck syndrome 2 which features underhydroxylation of lysine residues within telopeptides of collagen in the bone (Bank et al. 1999). Lysyl hydroxylase 3 (L5H-3) deficiency is caused by a mutation in the PLOD3 gene, and its clinical features are bone fragility with contractures, arterial rupture, and deafness (Salo et al. 2008).

Further hydroxylation reactions

Other members of the prolyl 4-hydroxylase family participate in pathways involving cellular response to hypoxia, such as hypoxia-inducible transcription factor (HIF)-P4H localized in the cytoplasm and catalyzes the hydroxylation of one or two proline residues in the oxygen-dependent degradation domain (ODDD) of HIF-α. The reaction requires the presence of molecular oxygen, and during normoxic conditions, the hydroxylated protein undergoes proteasomal degradation. In hypoxia, HIF-α evades degradation due to insufficient oxygen-dependent hydroxylation, binds to HIF-β, and fulfills its role as a transcription factor in the nucleus, activating more than 100 hypoxia-related genes (Myllyharju 2008).

A further member of the prolyl 4-hydroxylase family has an internal transmembrane domain (P4H-TM) and is localized on the surface of the ER membrane with its catalytic site facing into the ER lumen (Myllyharju 2008).

Ascorbate dependency of hydroxylation

Although the hydroxylation reactions catalyzed by the members of the hydroxylases are considered to be ascorbate dependent, the hydroxylation of the residues per se does not need ascorbate. Hydroxylases participate in two catalytic reactions: one in which the residues are hydroxylated, while 2-oxoglutarate is decarboxylated and molecular oxygen is

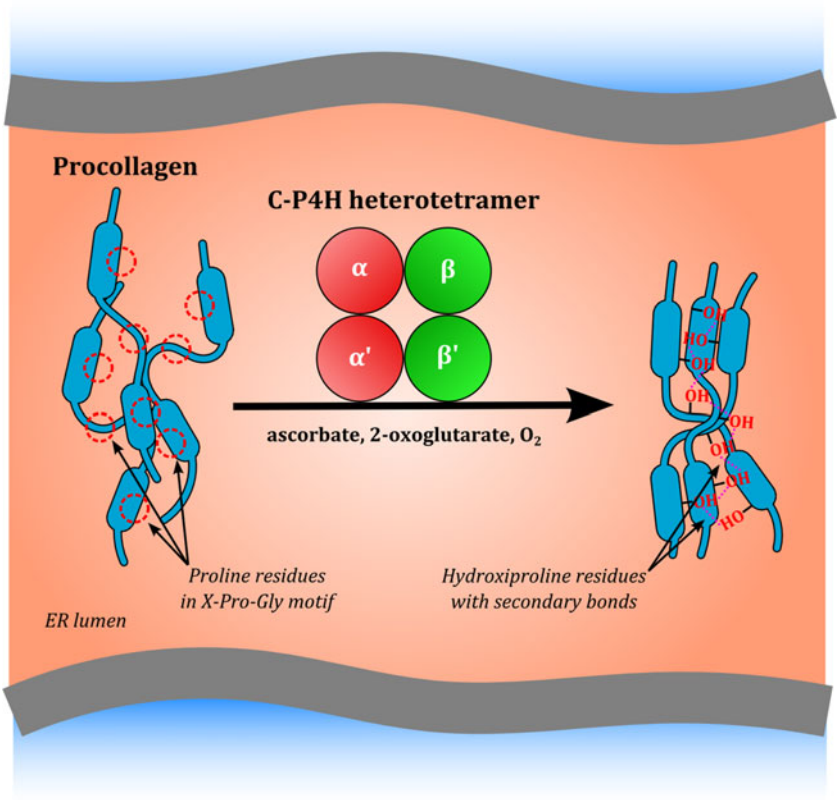

Fig. 1 Proline hydroxylation in the ER. Proline hydroxylation occurs at -X-Pro-Gly- triplets in a reaction catalyzed by collagen prolyl 4-hydroxylase (*C-P4H*), an $\alpha_2\beta_2$ heterotetramer localized in the ER lumen. The enzyme requires 2-oxoglutarate, oxygen, ferrous iron, and ascorbate for its function. The α subunit participates directly in the hydroxylation, and the β subunit of the complex was identified as protein disulfide isomerase

consumed (Fig. 2a). The atoms from molecular oxygen are incorporated into the hydroxyl group and the newly formed carboxyl group of succinate. This reaction is ascorbate independent. However, in another reaction, the hydroxylase catalyzes the decarboxylation of 2-oxoglutarate which is uncoupled from hydroxylation but still needs molecular oxygen (Fig. 2b, c). At this time, ferrous iron is oxidized and the protein is rendered catalytically inactive until reduced by ascorbate. Although the rate of the uncoupled carboxylation reaction is only 1–4 %, it takes place even with saturating concentrations of the substrate peptides; hence, ascorbate is essential to reactivate the oxidized enzyme (Myllylä et al. 1984; Kivirikko et al. 1989; Pihlajaniemi et al. 1991; Myllyharju 2003, 2008).

Furthermore, it has been reported that in the case of lysyl hydroxylase, ascorbate can be replaced by dithiothreitol and cysteine, but with a significantly lower reaction rate (Puistola et al. 1980).

Disulfide bond formation in the ER: initial steps

In addition to the hydroxylation of certain amino acid residues, ascorbate is involved in another protein folding reaction. The covalent bond formed between the thiol groups of cysteine residues is the strongest bond which stabilizes the tertiary structure of proteins. The formation of disulfide bonds was first investigated in the case of bovine ribonuclease by Anfinsen and his coworkers. In their classic experiments, they found that the fully reduced bovine ribonuclease would, under the influence of molecular oxygen alone, be oxidized to form an organized, enzymatically active product in high yield (White and Anfinsen 1959; White 1960). It has been concluded, therefore, that no special genetic information, beyond that contained in the amino acid sequence, is required for the proper folding of the molecule and for the formation of the "correct" disulfide bonds. However, during the further characterization of ribonuclease refolding reactivation, it was revealed that rat liver and pigeon pancreas homogenates contain a heat-sensitive, high molecular weight protein factor and a soluble low molecular weight, heat-stable, nonprotein factor, which together greatly accelerated the reactivation of reduced RNase, and each of which was inactive alone (Goldberger et al. 1963; Venetainer and Straub 1963). The protein part of this liver microsomal ribonuclease refolding, reactivating system was (partially) purified in the following year (Goldberger et al. 1964), and its official name PDI was first used in 1975 (Hawkins and Freedman 1975). The heat-stable soluble factor could be replaced by dehydroascorbic acid (DHA) in an in vitro refolding, reactivating experiment (Venetainer and Straub 1964). This was the first result which indicates that DHA is involved in the formation of disulfide bridges

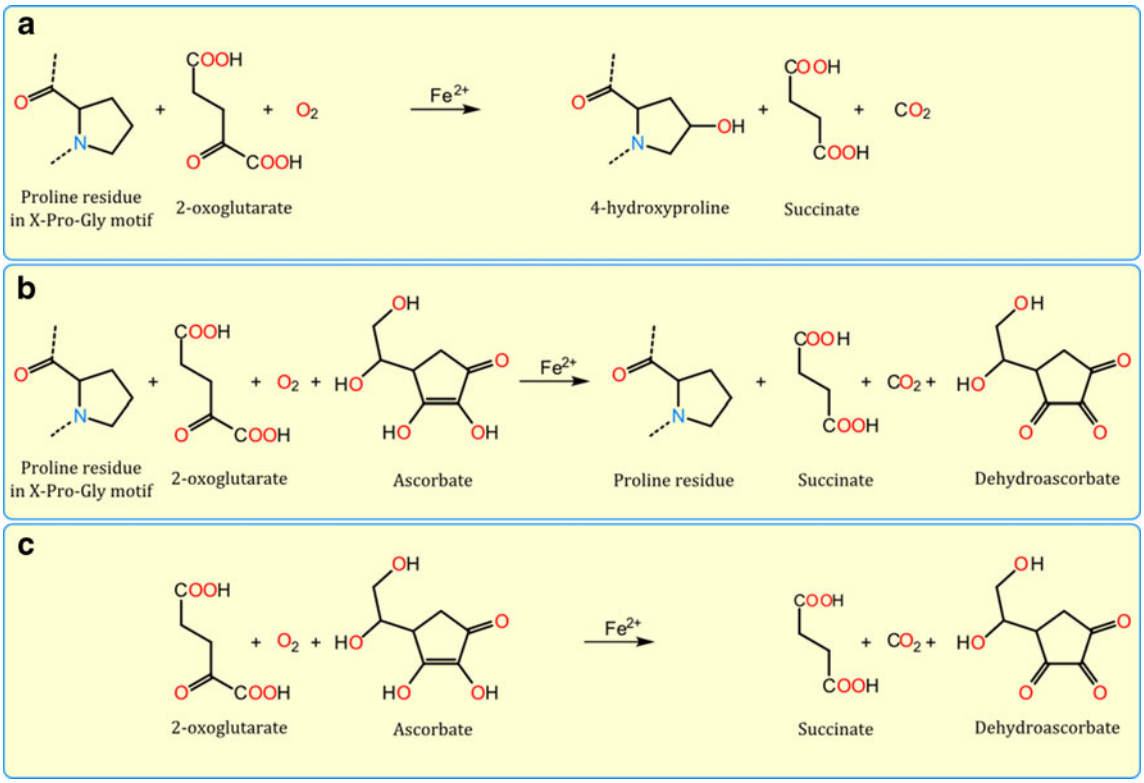

Fig. 2 **a**–**c** The reactions catalyzed by prolyl 4-hydroxylase. **a** The ascorbate-independent hydroxylation of proline residue with the concomitant decarboxylation of 2-oxoglutarate and consumption of molecular oxygen. The atoms from molecular oxygen are incorporated into the hydroxyl group and the newly formed carboxyl group of succinate. **b**, **c** The decarboxylation of 2-oxoglutarate uncoupled from hydroxylation of proline. This reaction still needs molecular oxygen. At this time, ferrous iron is oxidized, and the protein is rendered catalytically inactive until reduction by ascorbate. Ascorbate is stoichiometrically consumed in this uncoupled reaction, which may occur both in the presence (**b**) or absence (**c**) of the peptide substrate

(oxidative protein folding). The location of PDI was found to be the lumen of endoplasmic reticulum which is compatible with a model in which the enzyme behaves as a catalyst for the formation of native disulphide bonds in nascent and newly synthesized secretory proteins (Lambert and Freedman 1985). The sequence of rat PDI was determined, and it revealed two highly homologous regions with thioredoxin (Edman et al. 1985). The active site of PDI contains a thioredoxin-like CXXC motif with transitions between the dithiol disulfide states during the catalytic cycle of the enzyme. Since PDI oxidizes diverse ER substrate proteins by thiol–disulfide exchange, the CXXC motif of PDI should be reoxidized before the next catalytic cycle.

Dehydroascorbate as a potent electron acceptor

One candidate for the role of the electron acceptor can be the DHA since the DHA reductase activity of PDI was described a quarter century after the first involvement of DHA in disulfide bond formation. PDI reacted directly with DHA and GSH to catalyze the reduction of DHA to ascorbic acid with an apparent K_m, for DHA of 1 mM. The V_{max} was 8 nmol min^{-1} (Wells et al. 1990). Ascorbate addition to rat, guinea pig, or human liver microsomal vesicles resulted in a decrease in the protein thiol concentration, which was due to oxidation, since they could be reduced back with mercaptoethanol (Csala et al. 1999). Protein thiol oxidation could also be induced by DHA addition; however, its rate slightly (~30 %) lagged behind that induced by ascorbate. The addition of the ascorbate precursor, gulonolactone, to rat liver microsomes (a species that is capable of ascorbate biosynthesis) resulted in the oxidation of protein thiols almost to the same extent as in the case of ascorbate addition. The effect was totally absent in human or guinea pig liver microsomes, which do not have gulonolactone oxidase activity (Csala et al. 1999). Accordingly, the effect of gulonolactone addition can therefore be attributed to the formed ascorbate, i.e., to DHA. Since ascorbate must be oxidized to become an electron acceptor, this conclusion can be supported by the application of various cytochrome P450 inhibitors (econazole, proadifen, quercetin), which decreased both ascorbate consumption and protein thiol oxidation in liver microsomes (both from species capable and noncapable of ascorbate biosynthesis) upon ascorbate addition. Econazole inhibited the gulonolactone oxidase-dependent thiol oxidation without decreasing the rate of

ascorbate synthesis in rat liver microsomes (Csala et al. 1999). The effect of these inhibitors suggested the existence of a possible cytochrome P450-related ascorbate oxidase in the ER. Luminal ascorbate can be derived from biosynthesis and from transport through the ER membrane in ascorbate-synthesizing species. Exogenous ascorbate (or its oxidized form, dehydroascorbate) enters the ER from the cytosol in ascorbate-nonsynthesizing species (e.g., guinea pig and human). The transport of both ascorbate and dehydroascorbate through the membrane of the ER has been described (Bánhegyi et al. 1998), but the transport of ascorbate was found to be negligible, and the oxidation of ascorbate to dehydroascorbate was a prerequisite for the uptake (Csala et al. 2000). This observation indicates that ascorbate reaches the lumen of the ER in its oxidized form. In this way, dehydroascorbate (formed in or transported into the lumen of the ER) can be reduced by protein disulfide isomerase (Wells et al. 1990), oxidizing the active center CXXC motifs of the enzyme (Fig. 3).

The higher rate of DHA uptake and the significantly higher initial protein thiol content of hepatic microsomes prepared from spontaneously diabetic BioBreeding/Worcester rats were accompanied by a higher rate of dehydroascorbate reduction and increased protein thiol oxidation in microsomes from diabetic animals (Nardai et al. 2001). These findings further strengthened the oxidant role of DHA in the PDI-catalyzed protein disulfide formation.

Additional evidences were gained by the further characterization of the proposed ER-localized ascorbate oxidase. Sustained levels of ascorbyl free radical (AFR) and DHA could be maintained by the incubation of rat liver microsomal vesicles in the presence of cytosol like ascorbate concentration (Szarka et al. 2002). AFR generation with almost similar signal intensity and the appearance of measurable amounts of DHA could also be detected upon gulonolactone addition. AFR and DHA generation was proved to be heat and protease sensitive. The cytochrome P450 inhibitors (econazole and quercetin) applied earlier to inhibit both ascorbate consumption (oxidation) and protein thiol oxidation in liver microsomes (Csala et al. 1999, 2000) reduced effectively the intensity of the AFR signal both in the presence or absence of microsomes. Various metal chelators, such as 1, 10-phenanthroline or its copper-specific analogue neocuproine, reduced effectively the oxidation of ascorbate. Furthermore, these agents showed their inhibitory effect only in the presence of native microsomes. However, sodium azide was poorly effective; hence, the involvement of cytochrome P450s seems to be unlikely. All compounds which diminish ascorbate oxidation (AFR and DHA formation) inhibit protein thiol oxidation, demonstrating the connection between ascorbate oxidation and disulfide bond formation (Szarka et al. 2002). The copper-specific chelator, neocuproine (which inhibits enzymatic ascorbate oxidation more specifically), almost completely inhibited the ascorbate-dependent protein thiol oxidation even at very low concentrations (1 μM). The effect of the relatively copper-specific neocuproine (Al-Sa'doni et al. 1997) may suggest the participation of a copper oxidase, but the involvement of other metalloenzymes cannot be unequivocally excluded. However, it is worth noting that plant ascorbate oxidases are

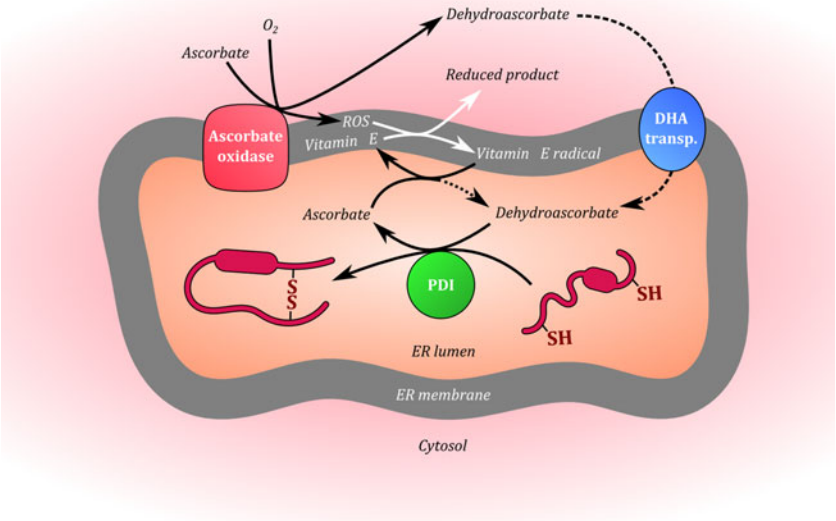

Fig. 3 The hypothesized role of DHA in oxidative protein folding. Cytoplasmic ascorbate can be oxidized by an ascorbate oxidase localized in the ER membrane with the concomitant production of H_2O_2 and other ROS. The generated DHA can reach the ER lumen with the mediation of a transporter. Tocopherol scavenges the ROS generated during the action of ascorbate oxidase. Tocopherol loses a hydrogen atom, and tocopheroxyl radical is formed. The tocopheroxyl radical is reduced by luminal ascorbate, while DHA is formed. Dehydroascorbate (formed in or transported into the lumen of the ER) can be reduced by PDI oxidizing the active center dithiols of the enzyme. Oxidized PDI reacts with reduced nascent proteins yielding protein disulfides and catalytically regenerating PDI

also cuproenzymes. Herewith, the missing component of DHA-dependent disulfide bond-forming machinery, a local ascorbate oxidase activity, was described (Szarka et al. 2002).

Since the ascorbate–thiol (glutathione) system is implicated in the reduction of the tocopheroxyl radical to tocopherol (Niki 1987), a role for vitamin E in the microsomal electron transfer pathway is plausible. Indeed, vitamin E deficiency partly uncoupled the two processes: ascorbate oxidation increased, while protein thiol oxidation decreased (Csala et al. 2001). The marked lipid peroxidation in microsomes from vitamin E-deficient rats indicates the accumulation of reactive oxygen species (ROS). The link between ascorbate and protein thiol oxidation could at least be partially restored by the in vitro addition of vitamin E to the deficient microsomes (Csala et al. 2001). All these observations demonstrate the direct role of vitamin E as a component of the protein thiol-oxidizing machinery in the hepatic endoplasmic reticulum transferring electrons from the thiol groups towards oxygen.

The above results can be summarized in the following in vitro model. Cytoplasmic ascorbate can be oxidized by an ascorbate oxidase localized in the ER membrane (using molecular oxygen) with the concomitant production of H_2O_2 and other ROS. The generated DHA can reach the ER lumen through the mediation of a transporter. Tocopherol scavenges the ROS generated during the action of ascorbate oxidase. Tocopherol loses a hydrogen atom and a tocopheroxyl radical is formed. The tocopheroxyl radical is reduced by luminal ascorbate, while DHA is formed. Dehydroascorbate (formed in or transported into the lumen of the ER) can be reduced by PDI oxidizing the active center dithiols of the enzyme. Oxidized PDI reacts with reduced nascent proteins yielding protein disulfides and catalytically regenerating PDI (Fig. 3).

This in vitro model was partially proved by in vivo experiments. Induction of ER chaperones (GRP78, GRP94) and PDI was observed in guinea pigs fed a scorbutic diet for 3 or 4 weeks (Margittai et al. 2005). Persistent ascorbate deficiency leads to ER stress, unfolded protein response, and apoptosis in the liver, suggesting that ascorbate participates in the general protein maturation, and its function is not restricted to procollagen synthesis.

Query about the role of ascorbate in oxidative folding

The above model seemed to be solid. However, Saaranen et al. (2010) found that the reaction of reduced PDI with DHA is probably too slow to be the major route for reduction of DHA in the ER. Not surprisingly, it is hard to predict an in vivo pathway from in vitro data. It should be noted that the intraluminal concentration of DHA is not known (neither the concentrations of other species). Although this observation does not mean that DHA is not linked to disulfide bond formation in the ER, but the above model should be handled with caution.

Redundancy in PDI reoxidation

At this point, we should take a short overview of the other PDI-oxidizing mechanisms in the ER. We will focus only on those most important points that are essential to understand the possible role of ascorbate in oxidative protein folding. Excellent reviews can be found on this topic (Sevier and Kaiser 2008; Margittai and Sitia 2011; Szarka and Bánhegyi 2011; Ramming and Appenzeller-Herzog 2012; Zito 2013).

PDI oxidizes diverse ER substrate proteins, while its CXXC motif becomes reduced, and it should be reoxidized before the next catalytic cycle. ERO1 described first in yeast (Frand and Kaiser 1998; Pollard et al. 1998) oxidizes, i.e., reactivates (CXXC motifs of) PDI. At least one ERO1 family member is present in all eukaryotes. While a single vital gene exists in yeast, two orthologs (ERO1α and ERO1β) were discovered in humans, with different tissue distributions and transcriptional controls (Cabibbo et al. 2000; Pagani et al. 2000). ERO1 by means of its cofactor flavin adenine dinucleotide transfers electrons to molecular oxygen, thus producing H_2O_2 (Tu and Weissman 2002).

The efficient disposal of H_2O_2 is done by means of ER peroxidases (Zito 2013). Three peroxidases were described in the mammalian ER: PRDX4 and the two GSH peroxidases GPX7 and GPX8 (Tavender et al. 2008; Nguyen et al. 2011). The in vivo interaction of both GPX7 and GPX8 with ERO1α has been described. In addition GPX7 significantly increased oxygen consumption by ERO1α, in vitro (Nguyen et al. 2011). Furthermore, it has been recently shown that GPX7 can utilize ERO1α-produced H_2O_2 to accelerate oxidative folding of substrates both in vitro and in vivo (Wang et al. 2013). PRDX4 is far the best characterized among the ER peroxidases. It has an outstanding reactivity with H_2O_2 (a second rate constant of $2.2 \times 10^7\ M^{-1}\ s^{-1}$) in comparison with the other ER peroxidases (Wang et al. 2012). This makes PRDX4 an efficient scavenger of low concentrations of H_2O_2. Peroxiredoxin dimer is formed during the peroxidase action of PRXD4. The oxidized PRDX4 is reactivated (reduced) by PDI which leads to the further insertion of disulfide bonds in new client proteins (Fig. 4). In this way, the cooperation between ERO1s and PRDX4 on one hand improves the efficacy of oxidative protein folding, by generating two disulfide bonds at the expense of one molecule of oxygen, and on the other hand, it solves the problem of hydrogen peroxide toxicity.

Both ERO1α and ERO1β seemed to be dispensable in viable mice (Zito et al. 2010). Although ERO1β-deficient animals are diabetic due to defects in insulin production and secretion and mice lacking ERO1α have an abnormal cardiac

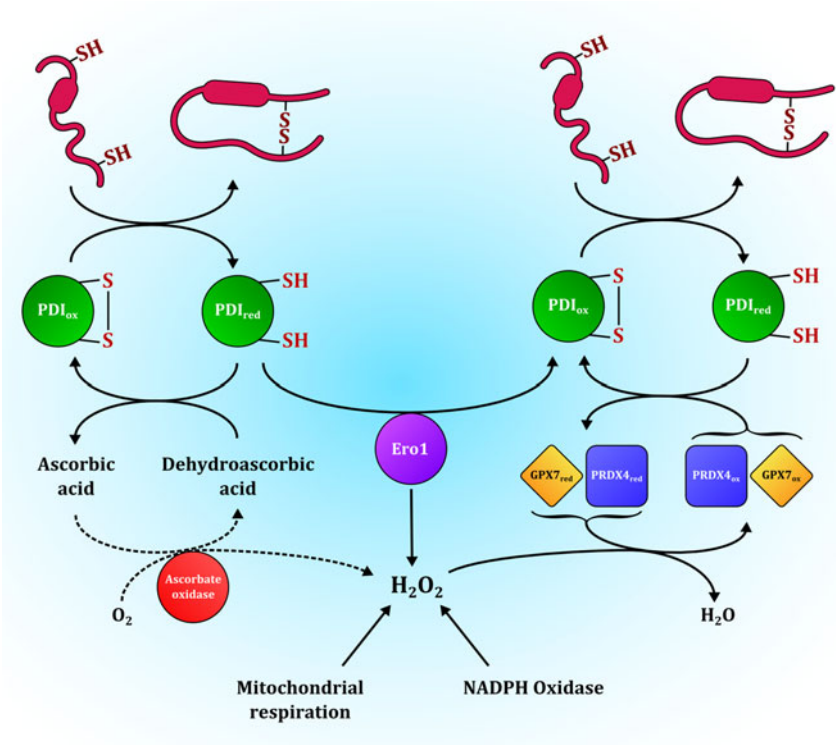

Fig. 4 ERO1s oxidize and, hence, reactivate (CXXC motifs of) PDI. ERO1s transfer electrons to molecular oxygen producing H_2O_2. The efficient disposal of H_2O_2 is done by ER peroxidases. The ER-resident peroxidases also promote the oxidative protein folding, directly utilizing H_2O_2 generated in the ER. In the absence of ERO1 activity, alternative sources of H_2O_2 can be the mitochondrial respiration and ER-localized NADPH oxidase(s). Furthermore, H_2O_2 can also be generated by the microsomal ascorbate oxidase enzyme (*dashed arrows*). This possible mechanism can get more significant role in the ERO1s and PRXD4 triple mutants and explains the observed depletion in ascorbate content

response to adrenergic stimulation (Chin et al. 2011; Zito et al. 2010), the other essential functions requiring disulfide bond formation remained unchallenged. Furthermore, PRDX4 null mice have a subtle defect in spermatogenesis, but there is no reported defect in metabolism of ER proteins (Iuchi et al. 2009). These relatively minor phenotypes can be explained by the existence of redundant mechanisms for thiol oxidation (Fig. 4).

The involvement of ascorbate in oxidative protein folding is reconfirmed

Interestingly, an unexpectedly modest delay in disulfide bond formation could only be observed in secreted proteins due to combined loss-of-function mutations in genes encoding the ER thiol oxidases ERO1α, ERO1β, and PRDX4 (Zito et al. 2012). Although this observation was a real surprise, it further demonstrated the redundancy in pathways to disulfide bond formation. However, this modest delay in disulfide bond formation was accompanied by a significant fivefold lower procollagen 4-hydroxyproline content and enhanced cysteinyl sulfenic acid modification of ER proteins. The reason of the decay in procollagen hydroxylation was also clarified. The tissue ascorbic acid content was lower in mutant mice. Furthermore, ascorbic acid supplementation could improve procollagen maturation. Since ascorbate can react with cysteinyl sulfenic acid side chains, the reduced ascorbate level mutant cells was explained by the accelerated clearance of ascorbate due to the elevated sulfenylated cysteines (Zito et al. 2012).

However, these observations can be interpreted in another way. The modest delay in disulfide bond formation of the triple mutants emphasizes the existence of further pathway(s). One candidate can be the aforementioned GPX7. This ER-resident peroxidase promotes oxidative protein folding, directly utilizing H_2O_2 generated in the ER. In the absence of ERO1 activity, alternative sources of H_2O_2 can be mitochondrial respiration and ER-localized NADPH oxidase(s). Furthermore, H_2O_2 can be generated by the aforementioned ascorbate oxidase enzyme (Fig. 4). This possible mechanism has a more significant role in the triple mutants and explains the observed depletion in ascorbate content. It can also explain the lower protein thiol oxidation ability of DHA in comparison to ascorbate (Csala et al. 1999).

Conclusions

Any of the aforementioned theories may be valid. It is an observed fact that the disturbance of the oxidative folding apparatus causes a disturbance of protein hydroxylation via ascorbic acid (Zito et al. 2012). Ascorbate represents a link between protein hydroxylation and protein disulfide bond formation in the ER. The elevated requirement of oxidative protein folding for ascorbate due to the diversion of electron

flow through ERO1s and PRDX4 causes low levels of ascorbate. This impairs the activity of proline 4-hydroxylase, proline 3-hydroxylase, and lysyl hydroxylase in the ER, which causes a delay in collagen maturation, the retention of procollagen in the ER, and consequently, a collagen-defective extracellular matrix that shares some aspects with scurvy. There are still many open questions on the role of ascorbate in disulfide bond formation. However, as recent results have shown, its role would be hardly disputed. The aforementioned triple mutant mice provide a good tool for the revelation of the details of its action. So, there is still a good chance of reading novel discoveries on the role of ascorbate in protein folding.

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Conflict of interest The authors declare that they have no conflict of interest.

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