

Ascorbate Oxidase, Protein Disulfide Isomerase, Ascorbic Acid, Dehydroascorbic Acid and Protein Levels in Developing Wheat Kernels and Their Relationship to Protein Disulfide Bond Formation

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ABSTRACT

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Developing wheat kernels of three New Zealand wheat cultivars at one to nine weeks postanthesis (WPA) were analyzed for kernel weight, salt-soluble protein, salt-insoluble protein, ascorbate oxidase (AOX), protein disulfide isomerase (PDI), ascorbic acid (AA), and dehydroascorbic acid (DHA). Kernel weight and salt-soluble protein increased from one to five or six WPA then declined. The amount of salt-insoluble protein per kernel rapidly increased from two to five or six WPA, then remained constant. AOX activity increased from one to two or three WPA then declined to almost zero at seven WPA. PDI activity increased from one to three

WPA, stayed constant to six WPA, then declined about 25%. DHA increased from one to two or three WPA then declined to zero at seven WPA. AA increased from one to three or five WPA then declined to zero at seven WPA. When DHA, at similar concentrations (≈ 1 mM) to that found in one to six WPA kernels, was mixed with reduced-gluten proteins, it was reduced to AA. The results are interpreted as a scheme showing that, during the formation of protein-disulfide bonds, electrons are transferred from nascent protein thiols, ultimately to oxygen, through a series of electron carriers including PDI, AA-DHA, and AOX.

The behavior of wheat flour in baking is largely influenced by the composition and structure of the proteins deposited in protein bodies during wheat development. There are many questions about the timing and biochemistry of protein folding, disulfide bond formation, and deposition. Protein disulfide isomerase (PDI) catalyzes the formation and rearrangement of disulfide bonds, but whether it does this to all gluten proteins throughout synthesis and deposition is uncertain (Kasarda 1999; Shewry 1999). Expression of PDI mRNA as a proportion of total RNA declines about two weeks postanthesis (WPA) (Grimwade et al 1996; DuPont et al 1998), but the presence of PDI protein in varying isoform patterns occurs for at least 45 days postanthesis (DuPont et al 1998; Skylas et al 2000). PDI activity has been detected in developing wheat kernels over 10–50 days postanthesis (Roden et al 1982), but there are no reports of quantitative enzyme activity measurements over the whole course of wheat kernel development. Intermolecular disulfide formation in SDS-insoluble glutenin seems to occur when gluten protein synthesis has ceased, ≈ 5 –6 WPA at the start of kernel dehydration (Aussenac and Carceller 2000). The role of PDI and other redox agents at this later stage of development is not clear, and one key question yet to be answered is the mechanism for oxidation of gluten thiols.

Despite many studies, the function of ascorbate oxidase (AOX) and dehydroascorbic acid (DHA) in plants is still poorly understood (Davey et al 2000). In animals, it has been proposed that DHA produced by a hypothetical oxidase at the surface of the endoplasmic reticulum oxidizes cysteines of PDI, which in turn oxidize nascent protein cysteines to form disulfide bonds (Wells et al 1993). Although this proposal is not supported by tests using insulin biosynthesis as a model (Wells et al 1995), there is evidence that DHA can act as suggested above (Givol et al 1964; Wells et al 1990; Nardai et al 2001).

In this article, we report the determination of the activities of PDI and AOX and the levels of DHA, ascorbic acid (AA), salt-insoluble gluten protein, and salt-soluble protein during development of wheat kernels. The distribution of AA and DHA within kernels, and the reduction of DHA by gluten protein thiols were also examined. The results are discussed in terms of the roles of AOX, PDI, AA, and DHA in protein disulfide bond formation and protein aggregation.

MATERIALS AND METHODS

Chemicals and Wheat Samples

Glutathione (>98%), L-ascorbic acid (99.7%), ribonuclease A, and cytidine 2':3'-cyclic monophosphate were purchased from Sigma Chemical Co. (St. Louis, MO). L-Dehydroascorbic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ascorbate oxidase (EC 1.10.3.3) from *Cucurbita* species was obtained from Boehringer Mannheim (Auckland, New Zealand). All other chemicals were of AnalaR standard, purchased from BDH Chemicals (Palmerston North, New Zealand).

In the spring of 1997, wheat (*Triticum aestivum*, 'Domino', 'Rata' and 'Karamu') was sown in 5.5-m² plots at the Crop & Food Research farm (Christchurch, New Zealand). Wheat heads (≈ 50 /plot) were tagged at anthesis on Monday 1 December for Domino, Monday 8 December for Rata, and Friday 28 November for Karamu. Exactly every week after anthesis up to nine weeks, five wheat heads were collected during the morning from each plot, frozen in liquid nitrogen, and stored at -80°C . Flour from mature Domino and Rata grain has strong dough and good breadbaking properties. Flour from mature Karamu grain has weak dough and poor breadbaking properties.

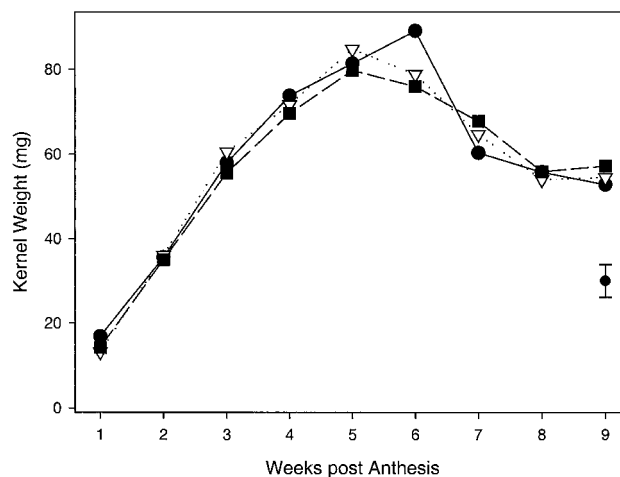


Fig. 1. Kernel wet weight of Rata (●), Domino (▽) and Karamu (■) during development of wheat. Each point represents the mean of five replicates. The bar is an average 95% confidence interval calculated from confidence intervals obtained from analyses of variance done for each week separately.

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Analytical Procedures

For each cultivar, five frozen kernels were taken from the middle portion of spikes, weighed, crushed, and extracted in 1.2M ammonium sulphate at pH 8.3 (1:3, w/v) for 40 min at room temperature. After centrifugation ($15,000 \times g$, 10 min), the supernatant was assayed for AOX (Every 1999) and salt-soluble protein (Bradford 1976). One unit (U) of AOX is defined as the amount of activity that oxidizes 1.0 μmol of AA in 1 min at 25°C and pH 6.2. The residue was analyzed for salt-insoluble protein (Gotham et al 1988). Protein content of kernels was calculated as mg/kernel (wet weight). Each sample was analyzed in duplicate, except weights, where five samples were measured.

Rough microsomes (endoplasmic reticulum) were prepared from ≈ 0.5 g of frozen kernels as described by Shimoni et al (1995) and assayed for PDI activity using reduced-ribonuclease A and cytidine 2':3'-cyclic monophosphate substrates as described by Lyles and Gilbert (1991). Reduced-ribonuclease was prepared as described by Grynberg et al (1977). One unit (U) of activity is defined as the amount of PDI producing an increase of one absorbance unit per minute at 25°C and pH 7.5. This rate was produced by a concentration of 14 nM active RNase in the assay mixture. Each sample was analyzed in duplicate.

For determination of AA and DHA distribution in kernels, seven frozen kernels from a sample of Domino at 3 WPA were partially thawed and dissected into two parts: endosperm and combined pericarp, seed coat, and embryo. Microsomes were prepared as described above. Frozen whole kernels, dissected kernel parts, and isolated microsomes were immediately ground in ice cold 1.33% perchloric acid (1:4, w/v) and analyzed for AA and DHA as described by Every (1996). Duplicate samples were analyzed.

To examine the role of protein thiols in reduction of DHA, samples of reduced gluten and reduced glutenin were prepared. Gluten was prepared by hand washing out starch and salt-soluble carbohydrates and proteins from a dough of wheat (Domino) flour. The gluten preparation was freeze-dried and ground to a powder. A glutenin-enriched fraction was prepared by extracting the gluten preparation with 70% ethanol in water (1:100, w/v) for 30 min at room temperature. Extracts were centrifuged ($5,000 \times g$ for 3 min), and the residue was freeze-dried and ground to a powder. This glutenin-enriched fraction will henceforth be called glutenin. Reduced proteins were prepared by mixing protein (0.5 g) with 0.5% dithiothreitol (25 mL) at pH 6.8 and 40°C for 1 hr. After centrifugation ($3,000 \times g$ for 5 min), the residue was washed five times with deoxygenated water (oxygen replaced with nitrogen). All samples were lyophilized and ground to a powder. Protein powder (30 mg) and 140 μL of 1.0 mM DHA in 50 mM phosphate and citrate buffer, pH 6.3, was mixed with a glass rod for various times. The reaction was stopped with chilled 5% perchloric acid and analyzed for AA and DHA (Every 1996). Each sample was analyzed in duplicate.

Kernel weight data (five replicates) were analyzed with analysis of variance using the GENSTAT statistical package. Because only duplicate samples were measured for all other tests, the results were presented graphically showing values for both duplicates.

RESULTS

In general, the accumulation patterns of kernel weight, protein, enzymes, AA, and DHA were similar for all three cultivars (Figs. 1 and 2). Wet kernel weight increased up to 5 WPA for Domino and

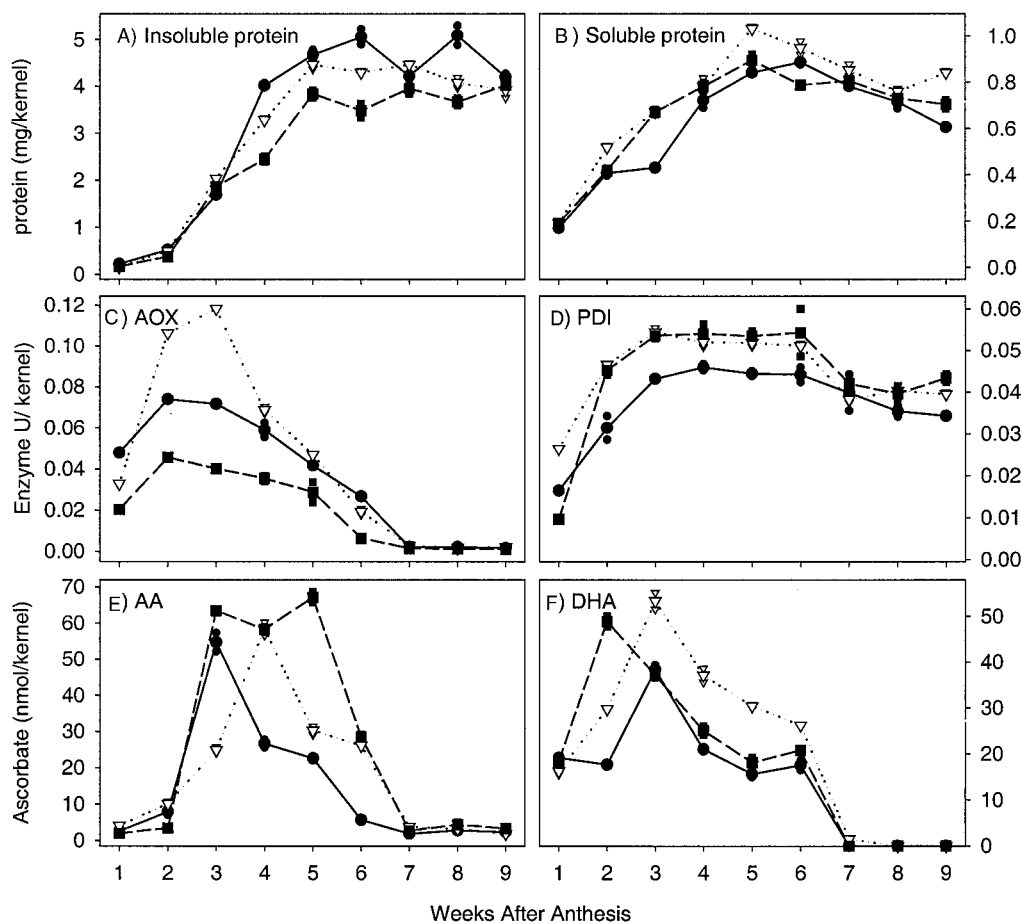


Fig. 2. Salt-insoluble protein content (A), salt-soluble protein content (B), AOX activity (C), PDI activity (D), AA content (E), and DHA content (F) in developing wheat kernels of Rata (●), Domino (▽), and Karamu (■). Kernels were measured as wet weight. Small symbols represent data for individual duplicates, large symbols are the means of the duplicates.

Karamu, and up to 6 WPA for Rata, after which time kernel dehydration and weight loss occurred (Fig. 1). For all cultivars, salt-insoluble proteins (Fig. 2A) and salt-soluble proteins (Fig. 2B) had accumulated to maximum levels about 5 WPA. Karamu is generally considered a low-protein cultivar, and at several stages during grain development there may have been slightly lower levels of salt-insoluble protein than the other cultivars (Fig 2A). The protein content of mature grain was 8.1% for Karamu, 11.8% for Rata, and 10.1% for Domino.

For all three cultivars, the activity of AOX peaked at 2–3 WPA (Fig. 2C), after which time activity decreased to almost zero by 7 WPA. For the samples tested, the three cultivars clearly differed in the maximum amount of AOX activity. PDI activity peaked at about 3 WPA and remained at maximum levels until 6 WPA (Fig. 2D), then declined slightly (possibly not significant) as kernels dehydrated and lost weight (Fig. 1).

The amount of AA per kernel increased to a maximum at 3–5 WPA (Fig. 2E), which was about a week later than the time DHA reached a maximum level (Fig. 2F). As for the levels of AOX in Domino, levels of DHA in Domino tended to be higher than the other cultivars. For the first 2 WPA, the level of AA in all cultivars was low and was less than the level of DHA; the mole fraction of AA was 10–30 mol% of the total ascorbate (AA plus DHA). For all cultivars, DHA levels were >16 nmol/kernel for the first 6 WPA (Fig. 2F), and then declined at the same time kernels dehydrated and lost weight (Fig. 1). The time DHA reached maximum level (2–3 WPA) coincided with maximum AOX and PDI activities and the time when salt-insoluble protein accumulation reached a maximum rate (Fig. 2A). Salt-soluble protein was accumulating at a maximum rate one week after anthesis (Fig. 2B).

In isolated parts of kernels from a bulk sample of Domino wheat at about 3 WPA, AA was detected mostly in the endosperm; the

remaining pericarp, seed coat, and embryo part of the kernel and the microsome fraction had about one-third the level of endosperm (Table I). High levels of DHA were detected in both the endosperm and the pericarp, seed coat, and embryo part of the kernel, whereas it was undetected in the microsome fraction.

Figure 3 shows the reduction of DHA to AA by reduced gluten and reduced glutenin. DHA was not reduced by nonreduced glutenin.

DISCUSSION

Currently, the main function of AOX and DHA is considered to be linked to reorganization of the cell wall because AOX is localized mainly in the cell wall apoplast and is most active in cell walls of fast-growing tissues such as germinating seeds, ovaries, and young fruits (Davey et al 2000). However, in fast-growing tissues there are also high rates of protein synthesis and disulfide bond formation in proteins destined for secretion or storage in protein bodies, as in developing wheat kernels (Grimwade et al 1996; DuPont et al 1998). In the three wheat cultivars tested in this study, the concentration of DHA (0.2–1.4 mM, on a mol/ wet weight of kernel) during the first six weeks of wheat kernel development (Fig. 2F) would provide a favorable oxidizing environment for formation of protein disulfide bonds. This period of high DHA concentration is the same period during which most disulfide bond formation occurs in gluten proteins (Gupta et al 1996; Panozzo et al 1996; Zhu and Khan 1999; Aussenac and Carceller 2000). Salt-insoluble wheat proteins, such as those shown in Fig. 2A, are well known to consist mainly of gluten proteins (Osborne 1907). The formation of intermolecular disulfide bonds in the largest glutenin polymers increases progressively during development to a maximum until grain maturity at 5–6 WPA (Zhu and Khan 1999). The albumins, globulins, and α - and β -gliadins appear to be synthesized mostly by 3–4 WPA (Gupta et al 1996; Zhu and Khan 1999), and the disulfide bonds formed in these proteins during this period would mostly be intramolecular. Salt-soluble proteins of wheat are well known to consist mainly of albumins and globulins (Osborne 1907), although under special circumstances, some gluten proteins can be extracted in salt solutions. The salt-soluble proteins shown in Fig. 2B would be expected to consist mainly of albumins and globulins, which would include the enzymes AOX and PDI that reached maximum levels at 2–3 WPA (Fig. 2C,D).

The chemical oxidation of protein cysteines to cystine (E'_{0} about –0.2 to –0.35 V) by DHA (E'_{0} = +0.058 V) is thermodynamically feasible. Indeed, DHA has been shown to chemically oxidize sulfhydryl groups in reduced-ribonuclease in vitro (Givol et al 1964; Venetianer and Straub 1964), and we have shown that reduced-gluten or reduced-glutenin proteins reduced DHA to AA (Fig. 3). Also, addition of DHA to a wheat flour dough yields AA evolution and an increase in glutenin polymer size over a control dough without DHA (Every et al 2000; Tsiami et al 2000). In developing wheat, it is also feasible that DHA oxidizes gluten cysteines to cystine because a

TABLE I

Distribution of Ascorbic Acid (AA) and Dehydroascorbic Acid (DHA) in Wheat Kernel of cv. Domino at Three Weeks Postanthesis

Kernel Part	AA ^a (nmol/g, wet wt)	DHA ^a (nmol/g, wet wt)
Whole kernel	444	724
Endosperm	758	880
Pericarp/seed coat/embryo	262	631
Microsome	203	0

^a Differences between duplicates were <10% of means, except for AA data of pericarp/seed-coat/embryo samples, which had a difference between duplicates of 18% of means.

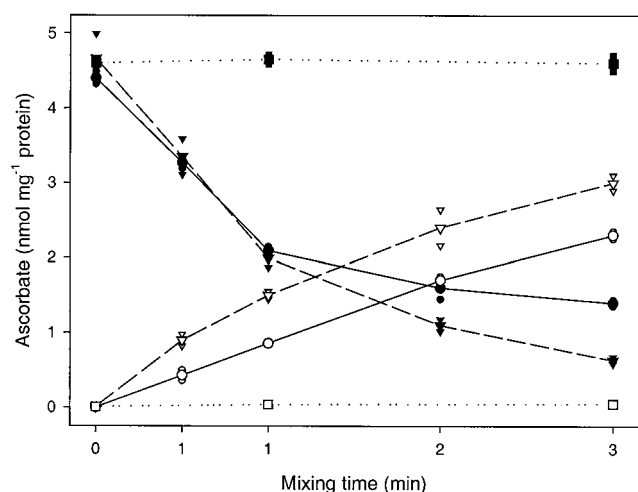


Fig. 3. Reduction of dehydroascorbic acid (solid symbols) to ascorbic acid (open symbols) by reduced gluten (●,○), reduced glutenin (▼,▽) and non-reduced glutenin (■,□). Small symbols represent data for individual duplicates, large symbols are the means of the duplicates.

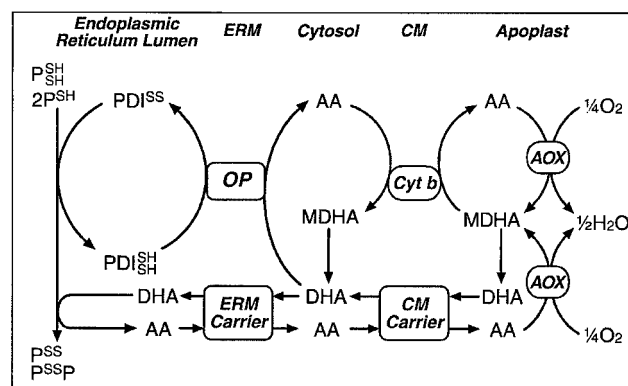


Fig. 4. Proposed pathways for protein disulfide bond formation in plants.

precise structural configuration of some wheat storage proteins would not be as necessary as for the formation of active enzymes where the action of PDI ensures functionally correct disulfide configuration. Doubt has been expressed about a role for PDI in gluten disulfide bond formation, especially intermolecular disulfide bonds in glutenin polymers (Kasarda 1999; Shewry 1999). Gupta et al (1996) reported that the accumulation of SDS-insoluble glutenin was completed by about 5 WPA. However, later work (Gobin et al 1997; Aussenac and Carceller 2000) indicates that the formation of intermolecular disulfide bonds in the large SDS-insoluble glutenin polymers mainly occurs at 5–6.5 WPA, during kernel dehydration and after these proteins have ceased synthesis at about 5 WPA. The mechanism of this reaction is not known. Because of the presence of DHA 5–6.5 WPA (Fig. 2F) during the period of kernel dehydration (Fig. 1) and after gluten proteins have ceased to accumulate at about 5 WPA (Fig. 2A), we suggest that during this period DHA directly oxidizes glutenin thiols to form intermolecular disulfide bonds in SDS-insoluble glutenin.

The doubts about the role of PDI in gluten aggregation arise because of the apparent early decline at 2 WPA of PDI-mRNA (Grimwade et al 1996; DuPont et al 1998) and PDI-protein (Shimoni et al 1995) relative to the period of disulfide formation in gluten, which occurs up to 7 WPA (Panozzo et al 1996; Gobin et al 1997; Aussenac and Carceller 2000). In these reports, however, the PDI-mRNA and PDI-protein levels were recorded as a proportion of total-mRNA and total-protein, and this represents an underestimation of their actual levels, because from about 2 WPA there is rapid accumulation of total-mRNA and total-protein. DuPont et al (1988) showed relatively high levels of PDI protein in endosperm and membrane fractions for at least 4 WPA, and Skylas et al (2000) reported PDI-protein, in varying isoform patterns, for at least 6 WPA. Roden et al (1982) detected PDI activity for at least 7 WPA, and we have demonstrated that the amount of PDI activity per kernel remained at maximum levels up to 6 WPA and then declined only $\approx 25\%$ 7–9 WPA (Fig. 2D). It is thus feasible that PDI is involved in formation of protein disulfide bonds throughout grain filling. PDI would certainly be necessary for the formation of functionally correct disulfide bonds in active secretory enzymes in the salt-soluble protein fraction shown in Fig. 2B, and the enzyme may be necessary for formation of some functionally correct disulfide bonds in the salt-insoluble gluten fraction (Fig. 2A). The results of Bulleid and Freedman (1988) suggest that PDI may be necessary for synthesis of correct intra-molecular disulfide bond configuration in γ -gliadin.

The standard redox potential of bovine liver PDI ($E'_{\circ} = -0.11$ V) (Hawkins et al 1991) makes it feasible for DHA to accept electrons from reduced PDI, either directly or indirectly through an electron carrier. Indeed, animal PDI has DHA reductase activity (Wells et al 1990; Nardai et al 2001) and appears to be more active in producing functionally correct disulfides in reduced-ribonuclease when in the presence of DHA (Givol et al 1964; Venetianer and Straub 1964). Protein disulfide formation occurs within the lumen of the endoplasmic reticulum, where PDI exclusively resides (Frاند et al 2000). Nardai et al (2001) reported that hepatic microsomes incubated with radiolabeled DHA accumulated DHA in the lumen of the endoplasmic reticulum. The increased isotope accumulation was accompanied by a higher rate of DHA reduction and increased protein thiol oxidation. In developing wheat kernels, high concentrations of DHA (≈ 1 mM) were detected in the endosperm but not in the isolated microsomes (Table I). DHA may have been present in the microsomes of intact kernels, however, but lost during the isolation procedure. Isolation took ≈ 4 hr at pH 8, in which time DHA and AA may have diffused from the microsomes. Also at the high pH, DHA may have been reduced by glutathione and protein thiols to AA, which was in fact detected in the microsomes (Table I).

In developing wheat kernels, the timing of AOX, PDI, AA, and DHA levels during the period of both salt-soluble and salt-insoluble protein accumulation (Fig. 2) and gluten-protein aggregation (Gupta et al 1996; Panozzo et al 1996; Zhu and Khan 1999; Aussenac and Carceller 2000) is consistent with the proposal shown schematically in Fig. 4. This scheme shows the involvement of AOX and AA/DHA,

with or without PDI, in the electron transport chain from protein thiols, ultimately, to oxygen, and may partly answer the long-standing question on protein folding “where do the electrons go?” (Glockshuber 1999). For proteins that require functionally correct disulfide bond configuration, electrons could flow from nascent protein thiols (P^{SH}) to PDI and then to a hypothetical oxidation protein (OP) in the endoplasmic reticulum membrane (ERM), which may be analogous to an oxidation protein, Ero1, that transfers oxidizing equivalents directly to PDI in yeast (Frاند et al 2000). Electrons could then flow from OP to DHA, either in the cytoplasm (shown in Fig. 4) or in the endoplasmic reticulum lumen (not shown in Fig. 4). If DHA is reduced to AA in the endoplasmic reticulum lumen, then the AA could be transported to the cytoplasm by facilitated diffusion using a hypothetical ERM-carrier that transports DHA or AA to or from the endoplasmic reticulum lumen. This carrier may be analogous to the hydrophobic, transporter-like protein encoded by a yeast HUT1 gene, which has genetic interaction with the yeast ERO1 gene (Nakanishi et al 2001). Electrons are then transferred from AA to oxygen through a *b*-type cytochrome (Cyt *b*) in the cytoplasmic membrane (Horemans et al 2000) (CM) and AOX in the apoplast. Alternately, AA may be transported to the apoplast by facilitated diffusion using a CM carrier (Horemans et al 2000) and there transfer its electrons to oxygen through AOX. For certain storage proteins that do not require precise disulfide bond configuration, electrons from protein thiols could flow directly to DHA. The resulting AA may be transported by carriers through the ERM and CM to transfer its electrons to oxygen through AOX, which catalyzes the oxidation of AA to monodehydroascorbic acid (MDHA).

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