

The Role of Dehydroascorbate in Disulfide Bond Formation

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Abstract

Dehydroascorbate (DHA) is a higher oxidation state of ascorbate formed through its action as an intracellular antioxidant. The recycling of DHA back to ascorbate is thought to be catalyzed by a variety of enzymes, including protein disulfide isomerase (PDI), linking ascorbate metabolism to oxidative protein folding in the endoplasmic reticulum (ER). Here we examine the possible role of PDI as a dehydroascorbate reductase. We find the reaction too slow to be the major route for reduction of DHA in the ER, with a second-order rate constant for the reaction of reduced PDI with DHA of only $12.5 \text{ M}^{-1} \text{ s}^{-1}$. Rates of a similar order of magnitude were obtained for other thioredoxin-superfamily members. However, glutaredoxin was able to catalyze DHA reduction more rapidly through a monothiol mechanism. In addition, DHA can rapidly react with many other dithiol systems, including dithiols in unfolded or partially folded proteins in a PDI-independent manner, with second-order rate constants of up to $186 \text{ M}^{-1} \text{ s}^{-1}$. Furthermore, we identify borate as a potent inhibitor of catalyzed and non-catalyzed DHA reduction *in vitro*. This study both provides insights into the link between ascorbate metabolism and oxidative protein folding and suggests a novel link between ascorbate metabolism and borate toxicity. *Antioxid. Redox Signal.* 12, 15–25.

Introduction

L-ASCORBATE, or vitamin C, is both an important intracellular antioxidant and an important cofactor in several metabolic processes, including prolyl-4-hydroxylation required for collagen biogenesis (see ref. 29 for review). It is a strong reducing agent with two physiologically relevant higher oxidation states, semidehydroascorbate (SDA) and dehydroascorbate (DHA) (Fig. 1A). The fate of these two species is very different. Two SDA radicals either can undergo a disproportionation reaction to form ascorbate and DHA or is recycled back to ascorbate through a number of reductases (see ref. 29 for review), whereas DHA is hydrolyzed rapidly at physiological pH (8) to form 2,3-diketogluconate. This hydrolysis, combined with the inability to produce ascorbate, means that humans require an efficient system for recycling DHA back to ascorbate.

The primary intracellular recycling system for DHA is thought to be based on its reaction with thiol groups to form disulfide bonds and ascorbate. Although the uncatalyzed reaction with cellular thiols such as glutathione is thought to be slow (50), the reaction in the cytoplasm is catalyzed by a variety of enzymes, especially glutaredoxins (see refs. 46 and 49 for *in vitro* characterization). Some of these small thioredoxin-superfamily members were originally identified as dehy-

droascorbate reductases, and DHA reductase activity along with catalysis of deglutathionylation reactions are still thought to be common traits for many glutaredoxin-family members.

The lumen of the ER is a more-oxidizing environment than the cytoplasm (20, 24), and many reactions that generate reactive oxygen species (ROS) occur there. These include hydrogen peroxide generation by Ero1 during disulfide bond formation (19) and L-gulonolactone oxidase action to generate ascorbate (9). Whereas other cellular compartments have many antioxidant enzymes that remove ROS, such as catalase and superoxide dismutase, many of these enzymes are absent from the ER or are present transiently *en route* through the secretory pathway (*e.g.*, ECSOD) (16). Hence, ascorbate is likely to function as a major antioxidant in the ER. This action generates DHA, but what happens to DHA generated in the ER? No ER resident glutaredoxins occur, although two novel Grx species are found in yeast that localize to *cis*-Golgi (31) and are transiently present in the ER. Currently it is thought that protein disulfide isomerase (PDI), one of the key enzymes in native disulfide-bond formation (for reviews, see refs. 2, 14, and 22), plays the role of a DHA reductase (49). Such a route not only would allow the regeneration of ascorbate, but the formation of oxidized PDI in this reaction also would allow it to feed into the process of native disulfide bond formation in

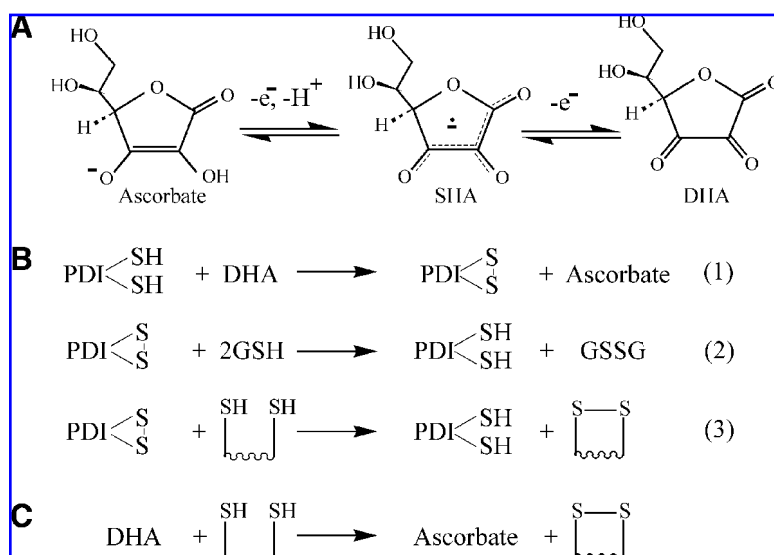


FIG. 1. Schematic representation of reactions involving DHA. (A) The oxidative states of ascorbate including dehydroascorbate (DHA) and semidehydroascorbate (SHA). (B) The reaction of reduced PDI with DHA to form a disulfide bond. The initial step is the oxidation of the active site of PDI and concomitant production of ascorbate (reaction 1). Oxidized PDI may react with GSH (reaction 2) or with dithiols in folding proteins (reaction 3), transferring the disulfide bond from PDI. (C) DHA may also react directly with dithiols in folding proteins, such as BPTI. The figure is adapted from ref. 22.

the ER (Fig. 1B). This would fit with data generated in the 1960s and early 1970s in which DHA was used as the net oxidant *in vitro* for disulfide bond formation (for examples, see refs. 18, 41, and 44). In addition, Bánhegyi and co-workers (6, 7), based on work using microsomes, repeatedly proposed a nonpassive role for DHA for disulfide bond formation *in vivo* (for examples, see refs. 6, 7, 11, 12, 34, and 42), but gaps are present in the model proposed (for example, the identity of the ascorbate oxidase in the system). Furthermore, the ability of PDI to act as a DHA reductase rests on a single publication (49), which lacks details of the probable mechanisms, and on whether the kinetics are fast enough to be physiologically relevant.

In this study, we examined the possible role of PDI as a DHA reductase. We 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. However, DHA is able to react rapidly with many other systems that contain at least two thiol groups, as long as both are accessible. The kinetics of these reactions, which include the reaction of DHA with dithiols in unfolded or partially folded proteins, are up to 15-fold faster than the reaction with the reduced active site of PDI. Because the reaction of DHA with unfolded proteins generates disulfides between spatially juxtaposed dithiol groups, this represents a potential PDI-independent method to use DHA in native disulfide bond formation in the ER. We also analyzed pathways for catalyzed and noncatalyzed DHA recycling *in vitro* and identified borate as a potent inhibitor of the recycling pathways, which may explain its toxicity in eukaryotic systems.

Materials and Methods

Protein expression and purification

Thioredoxin superfamily-member protein constructs used in this study were all previously cloned into an expression vector, which incorporates an N-terminal His-tag, for purification (1). Site-directed mutagenesis was performed according to instructions of the QuikChange kit (Stratagene, La Jolla, CA). All plasmids were checked for correctness by sequenc-

ing. Proteins were expressed in *Escherichia coli* strains BL21 (DE3) pLysS, except for Grx1, which was expressed in Rosetta-gami (Novagen, Madison, WI), and His-tagged proteins, were purified by immobilized metal affinity chromatography and ion-exchange chromatography, as described previously for the a domain of PDI (28). Pure protein fractions, as determined with Coomassie Brilliant Blue-stained SDS-PAGE, were combined and buffer exchanged into 20 mM sodium phosphate buffer, pH 7.3, and stored frozen.

Bovine pancreatic trypsin inhibitor (BPTI) was purified as described previously (27), and pure reduced BPTI was lyophilized and resuspended into 10 mM HCl, pH 2.0, to prevent oxidative refolding. RNase A (Sigma, St. Louis, MO) (1.7 mM) was reduced with DTT (700 mM) in 200 mM phosphate buffer containing 6 M guanidinium chloride and 1 mM EDTA for 2 h. DTT and guanidinium chloride were removed from RNase A by gel filtration by using Sephadex G-25 medium (Amersham Biosciences, Uppsala, Sweden) into 10 mM HCl, pH 2.0. The PDI substrate peptide, NRCSQGSCWN (37) was ordered from The Biomolecular Science Facility, Department of Biosciences, University of Kent (Canterbury, Kent, U.K.).

The concentrations of proteins and peptides used were calculated based on their molar-extinction coefficients at 280 nm (35).

Protein refolding

Protein refolding was initiated by the addition of denatured reduced protein to the refolding buffer (0.1 M sodium phosphate, 1 mM EDTA, pH 7.0). BPTI refolding was carried out at 50 μ M. When added, PDI was present at 7 μ M, and DHA (Sigma-Aldrich, Steinheim, Germany), at 0.5 mM. The folding reaction was stopped by the addition of 1.1 M iodoacetamide and BPTI, and its folding intermediates were purified with a PepClean C-18 spin column (Pierce, Rockford, IL) before ESI-mass spectrometry analysis (Micromass LCT, Manchester, U.K.). It should be noted that different species may bias their detection by ESI-mass spectrometry, and therefore, the results are only semiquantitative. The predicted

masses were 6,642.7 (three disulfide), 6,758.7 (two disulfide), 6,874.7 (one disulfide), and 6,990.7 (no disulfide). The experimentally determined masses for all samples were within 2 Da of the predicted masses.

Measuring the rate of reaction of DHA

The rate of noncatalyzed and enzyme-catalyzed reduction of DHA to ascorbate by reduced glutathione (GSH) was determined by measuring the increase in absorbance at 265 nm in 50 mM phosphate buffer, pH 7.3, including 0.5 mM EDTA and the desired amount of enzyme. Unless stated, the concentrations of GSH (Sigma-Aldrich) and DHA were 1 and 0.5 mM, respectively.

The direct rate of reaction of dithiol-containing reagents, including DTT, peptide, enzymes, and unfolded proteins, was determined by measuring the increase in absorbance at 265 nm in 50 mM phosphate buffer, pH 7.3, including 0.5 mM EDTA, 0.5 or 0.25 mM DHA, and the desired amount of reduced enzyme.

A molar extinction coefficient of $14,700 \text{ M}^{-1} \text{ cm}^{-1}$ at 265 nm for ascorbic acid (39) was used in the calculations.

When borate was used, a stock solution of 50 mM in 50 mM phosphate buffer was made, and the pH was adjusted to pH 7.3 to ensure no pH-dependent effects.

Because DHA is an unstable species, a 50 mM stock solution was made in 50 mM phosphate buffer, pH 7.3, and frozen in aliquots at -70°C . Each aliquot was used only on one day and was stored on ice after thawing. Some hydrolysis of the DHA will have occurred during preparation, and so the DHA concentrations were probably slightly lower than those calculated, although the rate of hydrolysis of DHA is slow (see Results section). Because the DHA solution did not have a strong absorbance at 265 nm, the maximal degree of possible contamination with ascorbate is low.

Results

MS analysis of BPTI refolding by DHA

To examine the hypothesis that the DHA-reductase activity of PDI could be linked to productive native disulfide bond formation in the ER, *in vitro* quantification of the relevant kinetics was examined.

The reaction of DHA with reduced PDI generates ascorbate and oxidized PDI. Oxidized PDI must in turn be reduced to complete the catalytic cycle (Fig. 1B). *In vivo*, one possible reactant for the second step is nonnative proteins, with the reduction of the active site of PDI being linked with the formation of a disulfide bond in the folding protein (Fig. 1B). To examine this reaction scheme, the refolding of the widely used model protein BPTI was analyzed. BPTI contains three disulfide bonds in the native state and has a complex folding pathway (10, 47, 48). With mass-spectrometric analysis as a rapid way to quantify the number of disulfide bonds in the different BPTI species as a function of time, BPTI refolding in the presence of 0.5 mM DHA and catalytic amounts of PDI was examined. The results (Fig. 2A) indicated that rapid refolding of BPTI to the native three-disulfide bond-containing state occurred, with 50% of the protein reaching the three-disulfide bond-containing state before the first time point at 2.5 min. This formation of the three-disulfide bond-containing state is rapid in comparison with refolding either

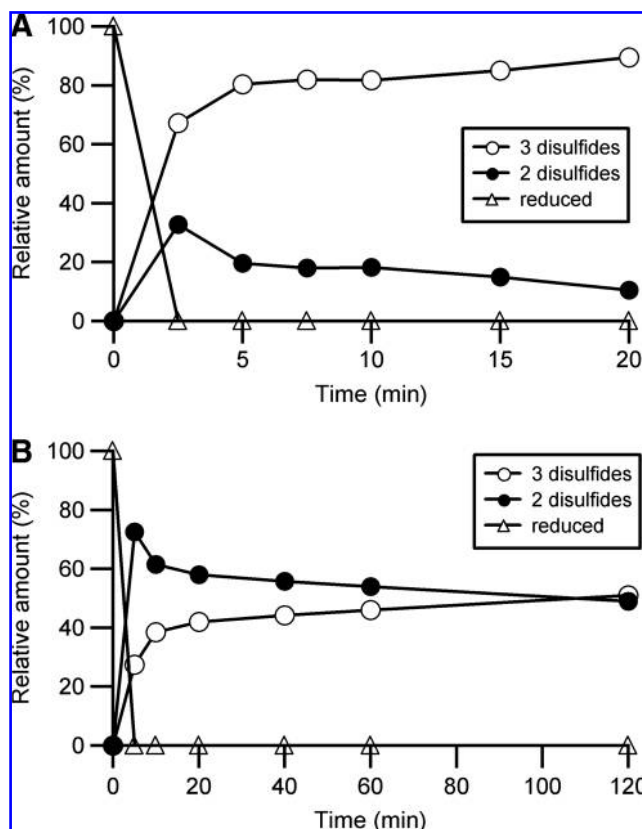


FIG. 2. Representative traces for the kinetics of BPTI oxidative refolding analyzed with electrospray mass spectrometry. (A) BPTI ($50 \mu\text{M}$) refolding in the presence of 0.5 mM DHA and catalytic amounts of PDI ($7 \mu\text{M}$). (B) BPTI ($50 \mu\text{M}$) refolding in the presence of 0.5 mM DHA. In the absence of an oxidant, no oxidative refolding of BPTI occurs with or without PDI present (data not shown).

in a glutathione buffer or with hydrogen peroxide as the oxidant, with PDI present in catalytic amounts (for example, see ref. 26). These *in vitro* results are consistent with the hypothesis that PDI is a DHA reductase and that DHA reduction by PDI can be linked to disulfide-bond formation in the ER.

Whereas these results can be taken as support the current hypothesis for the role of PDI, a control reaction must be examined. Analysis of a control reaction in which BPTI refolding was performed in the presence of 0.5 mM DHA, but in the absence of PDI, revealed that the noncatalyzed formation of disulfide bonds in BPTI by DHA (Fig. 1C) was also extremely rapid, with the complete formation of two disulfides by the first time point and subsequent slow formation of the three-disulfide-containing state (Fig. 2B). Separate analysis (see later) indicated that this slow formation of the three-disulfide state was not due to the loss of DHA by nonspecific hydrolysis, because under these buffer conditions, only $\sim 2.5\%$ of the DHA initially present hydrolyses over a 2-h period. These kinetics are reminiscent of the multiphase kinetics for the formation of the native three-disulfide state from the two-disulfide intermediates for the noncatalyzed refolding in a glutathione buffer (10, 47, 48). However, the formation of the two-disulfide state is at least 10-fold faster with 0.5 mM DHA than with a glutathione buffer [0.5 mM oxidized glutathione (GSSG) and 2 mM reduced glutathione (GSH)] in the same pH

7.0 buffer system (26). This is the fastest noncatalyzed formation of disulfide bonds in a folding protein of which we are aware. Because disulfide-bond isomerization is the rate-limiting step in BPTI refolding (10, 47, 48), the more-rapid formation of the three disulfide form in the presence of PDI may be due to the action of PDI as a disulfide isomerase rather than as a DHA reductase.

The noncatalyzed reactions

The unexpected rapidity of the noncatalyzed formation of disulfide bonds by DHA in a folding protein led us to re-evaluate whether PDI is actually a DHA-reductase. For both PDI and glutaredoxin, the DHA-reductase activity was defined by the ability to catalyze the reaction of DHA plus two GSHs to form ascorbate and GSSG (49). This use of GSH to reduce oxidized PDI may also be a physiologically relevant mechanism in the ER, because the reaction, when [GSH] is millimolar, is rapid (13), and the GSSG generated may in turn be used in disulfide-bond formation in folding proteins (see ref. 22 for arguments on the role of GSH and GSSG in disulfide-bond formation in the ER).

Before examining the ability of PDI to act as a DHA-reductase, the noncatalyzed rate for this reaction must be considered. The noncatalyzed, GSH-mediated reduction of DHA can easily be monitored by changes in absorbance at 265 nm, as ascorbate has a very significant absorbance at this wavelength, whereas DHA does not. A number of different published values exist for the molar extinction coefficient of ascorbate at 265 nm; we chose to use the value of $14,700 \text{ M}^{-1} \text{ cm}^{-1}$ (39) in all of the calculations, because this value gave $\Delta\text{absorbance}$ values that were consistent for the concentration of the dithiol species we subsequently investigated. Because the spontaneous hydrolysis of DHA is relatively fast, the change in absorbance for that was measured with varying DHA concentrations, and this change ($\Delta\text{Absorbance} = 5 \times 10^{-5}$ per mM of DHA per second) was taken into account in all measurements by subtracting it from the catalyzed rates. In all cases, the change in absorbance due to spontaneous hydrolysis of DHA was <2% of the total absorbance change.

The initial rate of reaction of 0.5 mM DHA with varying amounts of GSH (0.5–4 mM) showed a linear dependence of the rate on [GSH] with an implied second-order rate constant of $k_2 = 0.39 \pm 0.01 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 3A). In contrast, when the assay was done with a fixed GSH concentration and a varying DHA concentration, the initial rate was not linearly dependent on the concentration, but instead started to plateau at high concentrations of DHA (Fig. 3B). It is possible that DHA forms a transient complex with GSH, and when DHA is in excess over GSH, the free concentration of GSH is decreased, reducing the amount available to react with the GSH-DHA complex and hence reducing the overall rate of reaction.

PDI-catalyzed reactions

Consistent with previous results (50), the noncatalyzed rate of reaction of DHA with GSH to form GSSG and ascorbate is relatively slow, so a catalyst is likely to be present *in vivo* to accelerate the rate of DHA recycling. The initial rate of the PDI-catalyzed, GSH-mediated reduction of DHA was measured with varying concentrations of mature human PDI at fixed DHA and GSH concentrations (Fig. 4A). The initial rate

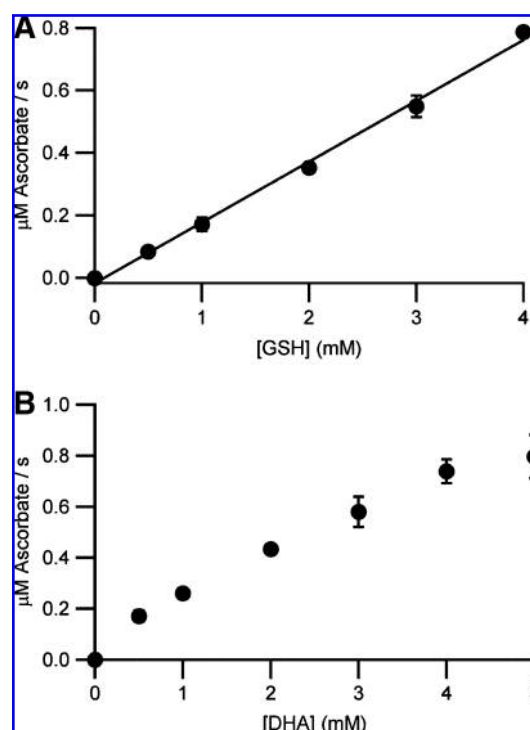


FIG. 3. Noncatalyzed reaction of DHA and GSH followed by changes in absorbance at 265 nm. (A) Linear dependence of the rate of formation of ascorbate with GSH concentration; [DHA] = 0.5 mM. **(B)** Dependence of the rate of formation of ascorbate with DHA concentration; [GSH] = 1 mM. The changes in absorbance due to hydrolysis are subtracted from both panels. The noncatalyzed rates are expressed as mean \pm SD, $n \geq 3$.

of ascorbate formation was linearly dependent on the PDI concentration, with $0.017 \mu\text{M/s}$ of ascorbate formed per micromole PDI. This gives a catalytic turnover of once per 59 s. This implies that under these conditions, PDI is a DHA reductase, but that it is very inefficient at catalyzing this reaction. Previously it was shown that the second-order rate constant for GSH reduction of PDI is $\sim 200 \text{ M}^{-1} \text{ s}^{-1}$ (13); hence, with 1 mM GSH present in the reaction, we would expect an initial rate of reduction of PDI by GSH of 0.2 per second. This would give a half-time of ~ 3.5 s for this reaction. This implies that the rate-limiting step in the action of PDI as a DHA reductase is the reaction of DHA with reduced PDI to form ascorbate and oxidized PDI. Because a catalytic turnover of about one per minute is very slow, and because it is unlikely that DHA concentrations will physiologically reach concentrations as high as 0.5 mM, it is unlikely that the DHA reductase activity of PDI is the major route for DHA reduction in the ER.

To confirm that the reaction of reduced PDI with DHA was the rate-limiting step in the DHA reductase activity of PDI, this reaction was directly examined by using the change in absorbance at 265 nm due to the formation of ascorbate. Reduced PDI was reacted with DHA in the absence of GSH and, as expected from the relative concentration of the species present, the reaction followed pseudo first-order kinetics, with $\Delta\text{absorbance}$ being proportional to the concentration of reduced PDI in the reaction, and the pseudo first-order rate

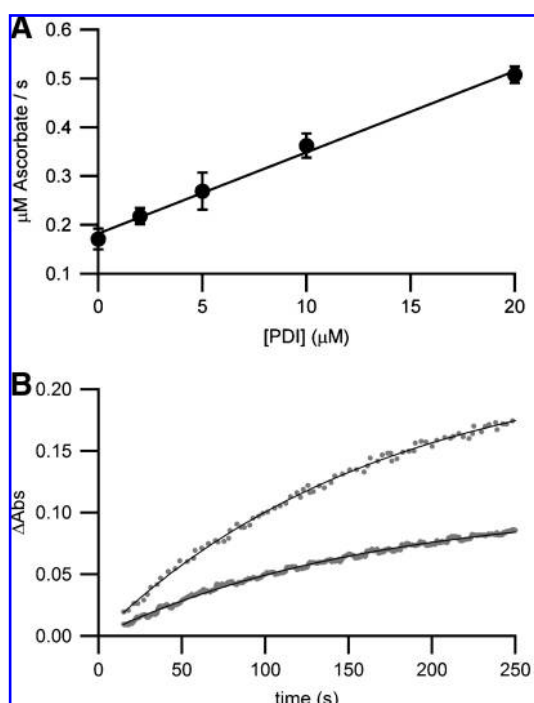


FIG. 4. The role of PDI in DHA reduction. (A) Linear dependence of the rate of formation of ascorbate with PDI concentration; [DHA] = 0.5 mM, [GSH] = 1 mM. The changes in absorbance due to hydrolysis are subtracted. The rates are expressed as mean \pm SD, $n \geq 3$. (B) Monitoring the rate of reaction of reduced PDI with DHA by changes in absorbance at 265 nm because of the formation of ascorbate. Upper trace, 10 μ M PDI; lower trace, 5 μ M PDI; [DHA] = 0.5 mM. The lines of best fit are to pseudo first-order reactions.

constant being independent of [PDI] but dependent on [DHA] (Fig. 4B). Analysis of these results implied a second-order rate constant for the reaction of PDI with DHA of $12.5 \pm 1.9 \text{ M}^{-1}\text{s}^{-1}$, thus confirming that the DHA oxidation of reduced PDI is the rate-limiting step in the action of PDI as a DHA reductase.

Other thioredoxin-superfamily members

PDI is the archetypal member of a family of ER-resident proteins (14, 22). Hence it is possible that although PDI may be unable to act with physiologically relevant kinetics as a DHA-reductase, another family member may be able to. To test this, five other human PDI-family members, along with four PDI mutants or domain constructs or both were purified and tested for their ability to catalyze the GSH reduction of DHA (Fig. 5A). The results indicated that although some of the other human PDI-family members were more active than human PDI in this assay, the fastest rate was only $0.050 \mu\text{M/s}$ of ascorbate formed per micromole ERp46, giving a catalytic turnover of once per 20 s. This is still probably too slow to be physiologically relevant, given the high concentration of DHA present in this *in vitro* assay.

The ability of three other thioredoxin-superfamily members to act as a DHA-reductase were then examined. *Escherichia coli* DsbA, a periplasmic enzyme involved in disulfide-bond formation, and *E. coli* thioredoxin 1 (Trx1), an enzyme involved in maintaining a reducing environment in the cytoplasm, both showed DHA-reductase activities be-

low those observed for PDI (Fig. 5A). In contrast, *E. coli* glutaredoxin 1 (Grx1) had an activity of $1.20 \mu\text{M/s}$ of ascorbate formed per micromole enzyme, giving a catalytic turnover of once per 0.8 s.

To examine whether this difference was due to the rate of reaction of the reduced enzyme with DHA to form the oxidized enzyme, the kinetics of DHA oxidation of the reduced active sites of Trx1 and Grx1 were directly measured by changes in absorbance at 265 nm due to the production of ascorbate. As per the reaction of reduced PDI with DHA, the reactions followed pseudo first-order kinetics, with Δ absorbance being proportional to the concentration of reduced enzyme in the reaction, and the pseudo first-order rate constant being independent of [enzyme] but dependent on [DHA] (see Fig. 5B and C). The second-order rate constants for the reaction of DHA with reduced Trx1 and Grx1 were similar ($k_2 = 26.3 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$ for Trx1, and $k_2 = 30.0 \pm 0.6 \text{ M}^{-1}\text{s}^{-1}$ for Grx1) and of the same order of magnitude as that of PDI. For Trx1, the result is consistent with the low DHA-reductase activity, but for Grx1, the rate of reaction of DHA with reduced Grx1 to form oxidized Grx1 is not consistent with a two-thiol mechanism, because the half-time for oxidation of Grx1 by 0.5 mM DHA is 46 s, and yet the enzyme has a catalytic turnover of once per 0.8 s. It was previously suggested that Grx1 may proceed through a mono-thiol mechanism whereby the second thiol group in the reaction comes not from the enzyme but from a bound molecule of GSH (46). If this theory is correct, then the C-terminal active-site mutant of Grx1 should be active as a DHA-reductase. This turns out to be the case, with the C14S Grx1 mutant being slightly more active than the wild-type enzyme, with a turnover of once per 0.6 s (Fig. 5A). In contrast, the C-terminal active-site mutant of the catalytic a domain of PDI, while retaining some DHA-reductase activity, has a reduced activity compared with the wild-type enzyme (Fig. 5A), suggesting that PDI acts through both a one-thiol (plus GSH) and a two-thiol mechanism. However, it should be noted that it uses both mechanisms inefficiently.

Other dithiol and monothiol systems

Whereas the one-thiol reagent GSH gave an apparent second-order rate constant for DHA reduction of $0.39 \text{ M}^{-1}\text{s}^{-1}$, three different dithiol-containing enzymes gave second-order rate constants of 12.5 to $30.0 \text{ M}^{-1}\text{s}^{-1}$. This difference may be due to monothiol *versus* dithiol reagents or due to specific features of the active sites of PDI, Trx1 and Grx1. To examine this, other dithiol and monothiol systems were tested in the assay. The monothiol reagents β -mercaptoethanol and cysteine behaved similar to GSH, with apparent second-order rate constants for the reaction of 0.17 ± 0.00 and $0.82 \pm 0.04 \text{ M}^{-1}\text{s}^{-1}$, respectively. The dithiol reagents DTT (see Fig. 6A and B) and a dicysteine-containing 10-amino acid peptide substrate for PDI activity (37) behaved similar to PDI, displaying pseudo first-order kinetics for reaction with DHA, with Δ absorbance being proportional to the concentration of dithiol species in the reaction, and the pseudo first-order rate constant being independent of [dithiol species] but dependent on [DHA]. The calculated second-order rate constants for the reaction with DHA were, however, much faster for DTT and the peptide than for PDI, Trx1, or Grx1, with values of 139 ± 4 and $186 \pm 14 \text{ M}^{-1}\text{s}^{-1}$, respectively.

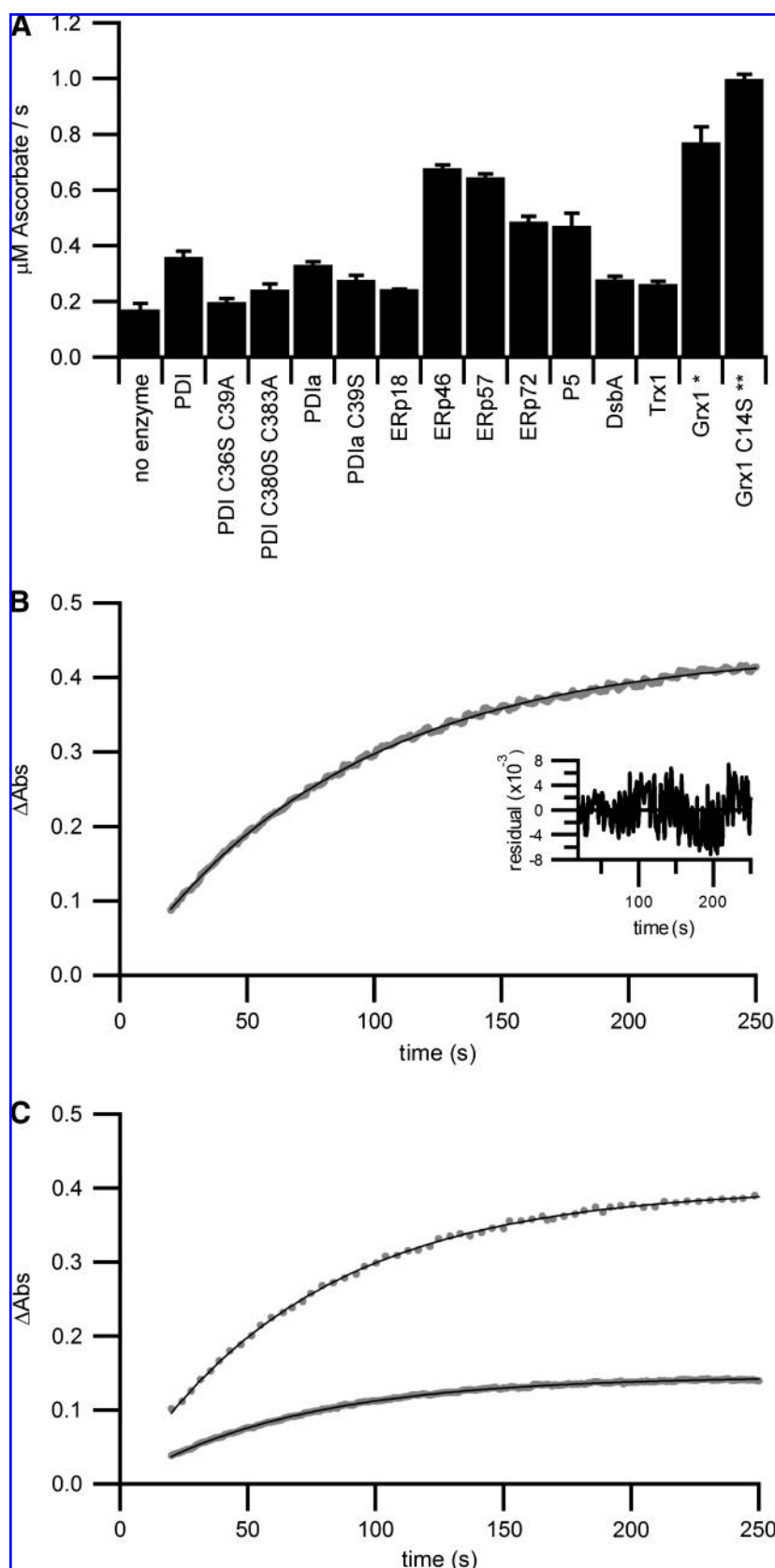


FIG. 5. The role of other thioredoxin-superfamily members in DHA reduction. (A)

The rate of catalytic ascorbate formation in the presence of different thioredoxin-superfamily members; [DHA] = 0.5 mM; [GSH] = 1 mM, [Enzyme] = 10 μM , except for [Grx1]* = 0.5 μM and [Grx1 C14S]** = 0.25 μM . The changes in absorbance due to hydrolysis are subtracted. The rates are expressed as mean \pm SD, $n \geq 2$.

(B) Monitoring the rate of reaction of reduced Trx1 with DHA by changes in absorbance at 265 nm due to the formation of ascorbate; [Trx1] = 30 μM , [DHA] = 0.5 mM. The line of best fit is to a pseudo first-order reaction, with the residuals shown in the insert.

(C) Monitoring the rate of reaction of reduced Grx1 with DHA by changes in absorbance at 265 nm due to the formation of ascorbate. Upper trace, 30 μM Grx1; lower trace, 10 μM Grx1; [DHA] = 0.5 mM. The lines of best fit are to pseudo first-order reactions.

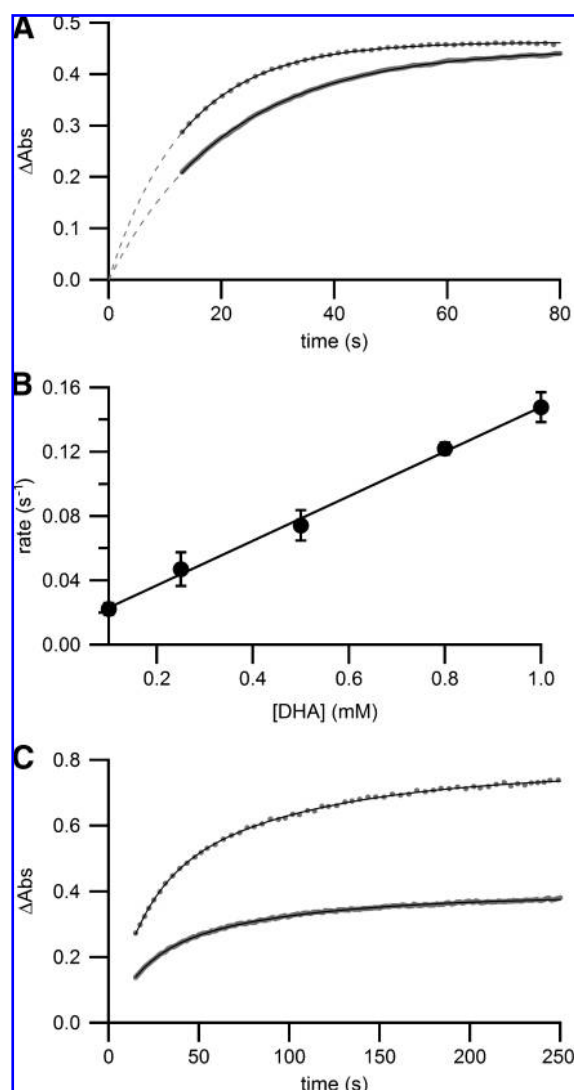


FIG. 6. The reaction of other thiol-containing species with DHA. (A) Monitoring the rate of reaction of DTT with DHA by changes in absorbance at 265 nm due to the formation of ascorbate. [DTT] = 20 μM ; upper trace, 0.5 mM DHA; lower trace, 0.25 mM DHA. The lines of best fit are to pseudo first-order reactions, with the *dashed lines* showing extrapolation of the lines of best fit back to $t=0$. (B) Linear dependence of the pseudo first-order rate constant for the rate of formation of ascorbate from the reaction of DTT with DHA; [DTT] = 20 μM . The rates are expressed as mean \pm SD, $n \geq 3$. (C) Monitoring the rate of reaction of reduced RNase with DHA by changes in absorbance at 265 nm due to the formation of ascorbate. Upper trace, 10 μM RNase; lower trace, 5 μM RNase; [DHA] = 0.5 mM. The lines of best fit are to a kinetic model with two sequential pseudo first-order reactions.

These results imply that the initial results observed are predominantly the result of monothiol *versus* dithiol reagents, and that, rather than having features that aid the reaction of the reduced active-site PDI, Trx1 and Grx1 actually react more slowly with DHA than do other dithiol systems. This lower reactivity may reflect the fact that the C-terminal active-site cysteine in the thioredoxin superfamily is not fully solvent exposed. Given the rapid rate of reaction of DTT and the

dithiol-containing peptide with DHA and the initial BPTI refolding results with DHA (Fig. 2B), we then decided to examine the rate of reaction of DHA with reduced unfolded proteins. Two protein substrates were chosen: BPTI, which contains three disulfides in the native state, and RNase, which contains four disulfides in the native state. Both of these are widely studied model proteins for examining disulfide-bond formation *in vitro* (for examples, see refs. 10, 47, and 48). For both proteins, the rate of reaction with DHA was monitored by the change in absorbance at 265 nm. The results for both BPTI and RNase (Fig. 6C) indicated that the reaction did not fit to a single pseudo first-order reaction. This is not surprising, because both proteins initially start in the more-flexible, fully reduced state, and as disulfide bonds are formed through the reaction with DHA, are likely to adopt a more-rigid structure, reducing access of the DHA to the remaining thiol groups and hence reducing the rate of reaction. Treating the formation of the individual disulfide bonds in each protein as a separate kinetic process resulted in several of the rate constants being equivalent. The simplest model for RNase refolding that gave random residuals was a two-step process, with each reaction being pseudo first order. The change in absorbance associated with each step was equivalent to the formation of two disulfide bonds, and the calculated second-order rate constants were 104 ± 9 and $22 \pm 1 \text{ M}^{-1}\text{s}^{-1}$. The kinetics for BPTI refolding were more complex. Fitting to a two-step process, with each step being pseudo first order and with changes in absorbance equivalent to the formation of two- and one-disulfide bonds, respectively, the calculated second-order rate constants were 163 ± 33 and $30 \pm 4 \text{ M}^{-1}\text{s}^{-1}$. Whereas the fit to the data by eye was good, for BPTI, close examination of the residuals to this fit revealed that they were not random, suggesting that the kinetics are more complex. This is consistent with the initial electrospray mass-spectrometric analysis (Fig. 2B) and with the concept of multiple folding pathways, with some two-disulfide-containing intermediates progressing very slowly to the native state (10, 47, 48).

Inhibition of DHA reduction by borate

A chance observation during these studies led us to realize that when DHA reduction by monothiol or dithiol reagents was studied in the presence of borate, the rate of reaction was significantly decreased. To study this effect further, the reaction of DHA with the dicysteine-containing PDI substrate peptide was analyzed by following changes in absorbance at 265 nm as ascorbate is formed. At constant pH but with increasing concentrations of borate present in the system, the pseudo first-order rate of reaction decreased, but with no apparent change in the $\Delta\text{absorbance}$ (Fig. 7A). At high concentrations of borate (e.g., 0.1 M), no reaction between DHA and the peptide could be observed (no reaction could be observed between DHA and other thiol-containing reagents, either). By varying the concentration of borate, a K_i value of $0.60 \pm 0.04 \text{ mM}$ for the reaction of 0.25 mM DHA with 10 μM peptide was obtained (Fig. 7B).

Discussion

For the past decade, it has been proposed that DHA may be linked to native disulfide-bond formation in the ER (6, 7, 11, 12, 34, 42). This proposal did not receive the full

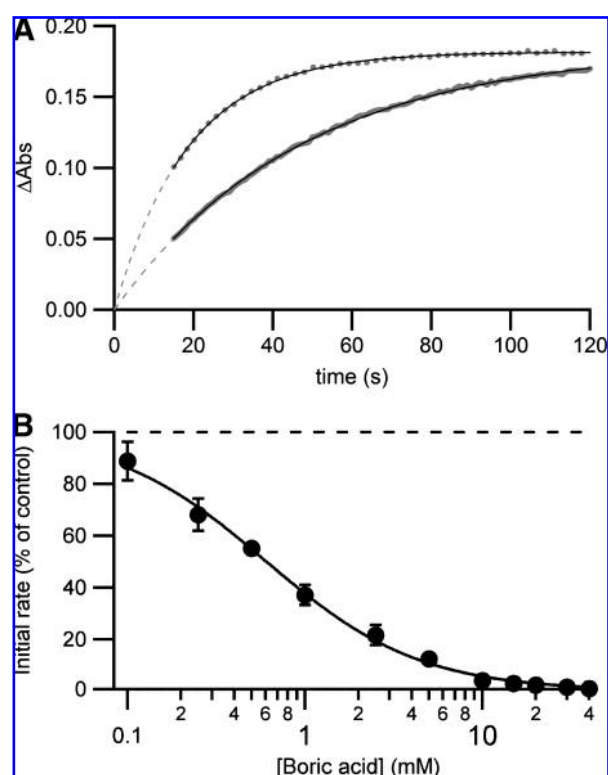


FIG. 7. The inhibition of the reaction of DHA with a dicysteine-containing decapeptide by borate. (A) Monitoring the rate of reaction of the peptide with DHA by changes in absorbance at 265 nm due to the formation of ascorbate. [Peptide] = 10 μM ; [DHA] = 0.25 mM; upper trace, no borate; lower trace, 1 mM borate. The lines of best fit are to pseudo first-order reactions. (B) A plot of the initial rate of reaction of the peptide and DHA, as a percentage of the control, with borate concentration; [peptide], 10 μM ; [DHA], 0.25 mM; pH 7.3 in all reactions. The initial rates are expressed as mean \pm SD, $n \geq 2$, and the calculated K_i is 0.60 mM.

attention it deserved, in part because of the timing against the high-profile reporting of the role of the Ero1 family (for examples, see refs. 17, 19, and 36). In part, this hypothesis arose as it had been reported that PDI, a principal component of disulfide-bond formation in the ER, also had DHA-reductase activity (49). However, to our knowledge, more-detailed studies on the kinetics of this activity and whether they are fast enough to be physiologically relevant have not been undertaken.

The data presented here suggest that although PDI has DHA-reductase activity, it is relatively inefficient. Dissection of the process of DHA reduction by PDI revealed that the rate-limiting step was oxidation of reduced PDI by DHA to generate oxidized PDI and ascorbate, with a second-order rate constant of $12.5 \text{ M}^{-1} \text{ s}^{-1}$, whereas GSH reacted with DHA with a second-order rate constant of $0.39 \text{ M}^{-1} \text{ s}^{-1}$. It is problematic to deduce *in vivo* routes from *in vitro* data, especially because the intraluminal concentrations of the species are not known. However, given that it is probable that GSH is present at least in 100-fold in excess over reduced PDI in the ER lumen, whereas the difference in rates is only 32-fold, it is likely that PDI is not directly involved in the major *in vivo* route for

DHA reduction, unless it is in complex with the source of DHA production (*i.e.*, that the local concentration of reactant species is very high). However, this result does not mean that DHA is not linked to disulfide-bond formation in the ER. Whereas PDI and other PDI-family members react relatively slowly with DHA, glutaredoxin can catalyze DHA reduction by a one-thiol mechanism (46 and data presented here), and two one-thiol glutaredoxins were recently reported in the early secretory pathway of the yeast *Saccharomyces cerevisiae* (25, 31), although we can find no mammalian homologues, suggesting this cannot be a general mechanism. In addition, other dithiol-containing species can react much faster with second-order rate constants of up to $186 \text{ M}^{-1} \text{ s}^{-1}$. These more-reactive species include nonnative proteins and peptides. Although the concentrations of such species in the ER-luminal is unknown and probably highly variable between cell types, a recent elegant study by Hansen and co-workers (21) pointed out that the total intracellular protein thiol pool is significantly greater than that of the intracellular GSH pool and hence may make a significant contribution to protection against redox reagents. Overall, our results suggest that the antioxidant defense in the ER may be linked directly to disulfide-bond formation in folding proteins in the ER without PDI as an intermediary.

Whereas DHA may be generated by the reaction of ascorbate with ROS, or potentially through a mechanism to transport of ascorbate into the ER (7, 12, 34), no evidence exists to date for the active formation of DHA to make disulfide bonds. In addition to the generation of DHA from ascorbate by the passive reaction of ascorbate with ROS, active processes for catalyzing this reaction are found in some organisms, based on the action of ascorbate oxidases. L-Ascorbate oxidases (EC 1.10.3.3) are copper-dependent enzymes made in plants that contain plastocyanin-like domains and catalyze the oxidation of L-ascorbate to dehydroascorbate by using molecular oxygen. Although these N-glycosylated enzymes fold in the ER, they are thought to be secreted. Similarly, the four human enzymes that are known in the multicopper oxidase family also are extracellular proteins; ceruloplasmin and hephaestin are involved in iron transport (23, 45), whereas coagulation factors V and VIII are involved in hemostasis (40). No evidence exists for an active process to make disulfide bonds in the ER through the oxidation of ascorbate to DHA. Furthermore, because eukaryotic organisms have a number of ER-located sulfhydryl oxidases [for example, the Ero1 family (17, 36) or members of the ERV/ALR sulfhydryl oxidase domain containing sulfhydryl oxidases (43), such as ERV2 (38)], the question arises as to why have an additional active parallel pathway? Hence, disulfide bond formation in the ER through the action of DHA probably does occur, but by passive processes linked either to the role of ascorbate as an antioxidant or to ascorbate uptake into the ER (7, 12, 34).

Although the focus of this study was on the potential role of PDI as a DHA-reductase, the results have implications for ascorbate recycling in other cellular compartments. Our results indicate that the reactions of dithiol systems with DHA are much more efficient than are the use of monothiol systems, presumably because of the stability of the intermediate species. However, the dithiol need not come from a single species, with the efficient DHA-reductase activity of *E. coli* Grx1 coming from a single thiol on the enzyme combined with a

thiol from an enzyme-bound GSH. Currently, two major pathways are thought to exist for DHA reduction in the cytoplasm: reduction coupled to thioredoxin/thioredoxin reductase as well as the action of glutaredoxin/glutathione coupled to glutathione reductase. With multiple pathways, the physiological contribution of any of these pathways is difficult to determine. However, our results indicate that the oxidation of reduced Trx1 or Grx1 by DHA, although significantly faster than the reaction of DHA with GSH, is a relatively slow event, with second-order rate constants of only $26.3 \text{ M}^{-1} \text{ s}^{-1}$ for Trx1 and $k_2 = 30.0 \text{ M}^{-1} \text{ s}^{-1}$ for Grx1. With 0.5 mM DHA, this gives a half-time for the reaction with reduced Trx1 of nearly 1 min, but the concentration of DHA is unlikely to become this high under physiological conditions. In contrast to Trx1, Grx1 does not use this mechanism; instead, the utilization of a single thiol on the enzyme and a thiol from enzyme-bound GSH means that the catalytic turnover can be on the subsecond timescale and that one-thiol glutaredoxins may be as efficient, or even more so, than two-thiol family members. Hence, it is likely that the reduction of DHA by glutaredoxin/glutathione is the major route *in vivo* unless the DHA reacts with dithiols in other proteins in the high-intracellular-protein thiol pool (21), generating an intramolecular protein disulfide bond that would be reduced by thioredoxin. Both routes, however, ultimately consume one NADPH per DHA reduced, and the metabolically efficient linkage of DHA reduction with disulfide-bond formation in folding proteins that probably occurs in the ER may also occur in other cellular compartments in which disulfide-bond formation occurs (for example, in the intermembrane space of mitochondria).

Our observation that borate is a potent reversible inhibitor of the reduction of DHA also has implications for other systems. Borate is known to be toxic to eukaryotic systems. It inhibits proliferation of cancer cells with a 50% inhibition value close to our calculated K_i value (3–5); concentrations $>270 \mu\text{M}$ are associated with fetal stunting in rats (15); and reduced crop yields due to toxic levels of boron are a major problem in food production (33), with 10 mM boric acid being “substantially lethal” to wild-type *Arabidopsis* (32). The lethality of borate is thought to be associated with the interaction between borate and the *cis*-diols on five-carbon furanose sugars, in particular, ribose in ribonucleotides and apiose in the plant cell wall (30). To our knowledge, borate toxicity has not been linked to ascorbate metabolism, but given the similarities in the chemical structures of ascorbate and DHA with the furanose sugars and our results on the strong inhibition of the reduction reaction of DHA, we suggest that borate toxicity may also be linked to ascorbate metabolism.

Finally, the use of DHA as a reagent to form disulfide bonds *in vitro* should perhaps be reevaluated. Although it was used in several landmark articles >40 years ago (for example, see refs. 18 and 44), it has been overtaken by the use of glutathione buffers and all but forgotten. However, the second-order rate constant for the formation of the first two disulfide bonds in BPTI is $163 \text{ M}^{-1} \text{ s}^{-1}$, >20 -fold faster than that in a glutathione-redox buffer or by using hydrogen peroxide as the oxidant (26). Furthermore, the reaction can be efficiently quenched in a reversible manner by the addition of borate, a far more convenient method than those available when using other oxidants.

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Author Disclosure Statement

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Abbreviations Used

BPTI = bovine pancreatic trypsin inhibitor
DHA = dehydroascorbate
DTT = dithiothreitol
EDTA = ethylenediaminetetraacetic acid
ER = endoplasmic reticulum
Grx = glutaredoxin
GSH = reduced glutathione
GSSG = oxidized glutathione
HPLC = high-performance liquid chromatography
PDI = protein disulfide isomerase
ROS = reactive oxygen species
SDA = semidehydroascorbate
TFA = trifluoroacetic acid
Trx = thioredoxin

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