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Dehydroascorbate Reduction

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Dehydroascorbic acid is generated in plants and animal cells by oxidation of ascorbic acid. The reaction is believed to occur by the one-electron oxidation of ascorbic acid to semidehydroascorbate radical followed by disproportionation to dehydroascorbic acid and ascorbic acid. Semidehydroascorbic acid may recycle to ascorbic acid catalyzed by membrane-bound NADH-semidehydroscorbate reductase. However, disproportionation of the free radical occurs at a rapid rate, $10^5\ M^{-1}\ s^{-1}$, accounting for measurable cellular levels of dehydroascorbate. Dehydroascorbate reductase, studied earlier and more extensively in plants, is now recognized as the intrinsic activity of thioltransferases (glutaredoxins) and protein disulfide isomerase in animal cells. These enzymes catalyze the glutathione-dependent two-electron regeneration of ascorbic acid. The importance of the latter route of ascorbic acid renewal was seen in studies of GSH-deficient rodents (Meister, A. (1992) *Biochem. Pharmacol.* **44,** 1905–1915). GSH deficiency in newborn animals resulted in decreased tissue ascorbic acid and increased dehydroascorbate-to-ascorbate ratios. Administration of ascorbic acid daily to GSH-deficient animals decreased animal mortality and cell damage from oxygen stress. A cellular role is proposed for dehydroascorbate in the oxidation of nascent protein dithiols to disulfides catalyzed in the endoplasmic reticulum compartment by protein disulfide isomerase.

KEY WORDS: Dehydroascorbic acid; ascorbic acid; semidehydroascorbic acid; glutathione; glutathione: dehydroascorbate oxidoreductase; NADH semidehydroascorbate reductase; L-buthionine-SR-sulfoximine; gastric ascorbate; protein disulfide bonds; protein disulfide isomerase.

INTRODUCTION

Dehydro L-ascorbic acid (DHA), the first chemically stable product of L-ascorbic acid (AAH_2) oxidation, is generated in plant or animal cells from L-ascorbic acid by one- or two-electron oxidation reactions. Semidehydroascorbic acid ($^{\bullet}AAH$) may or may not be a required intermediate. Autoxidation of ascorbic acid in water at near neutral or alkaline pH is mediated by $O_2^{\bullet -}$ generated by transfer of an electron to dioxygen from a donor such as a transition metal. In support of this theory, autoxidation of ascorbic acid is inhibited by chelators and superoxide dismutase (SOD) (Puget and Michaelson, 1974). Ascorbic acid was known for many years to be easily oxidized by halogens (Pecherer, 1951), oxygen (Ohmori and Takagi, 1978), quinones (Sjostrand, 1970), H_2O_2 (Szent Györgyi, 1928), etc., to dehydroascorbic acid. Dehydroascorbic acid is found in most biological tissues at low levels compared with a relatively high ascorbic acid content (Tolbert and Ward, 1982).

The presence of DHA in plant and animal cells points to the existence of persistent oxidation reactions and to the necessity of a cell to regenerate ascorbic acid continuously. This necessity is the result of essential functions of ascorbic acid including its antioxidant capacity, and its cofactor roles in many enzyme systems, e.g., prolyl 4-hydroxylase (Myllylä *et al.*, 1978), γ-butyryl betaine hydroxylase (Lindstedt and Lindstedt, 1970), and dopamine β-hydroxylase (Levine *et al.*, 1985). These reactions lead to the production of semidehydroascorbate and ultimately dehydroascorbate.

Given the importance of ascorbate in cellular functions, it is of current biological interest to detect

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the factors involved in the mechanism of cellular ascorbate regeneration. The reduction of dehydro-L-ascorbate by glutathione (GSH) is a well-documented chemical reaction (Borsook *et al.*, 1937). Nevertheless, the extent to which the catalyzed and noncatalyzed reactions occur, *in vivo*, is unknown. An enzyme, glutathione : dehydroascorbate oxidoreductase (EC 1.8.5.1), also called DHA reductase, was described several decades ago in plants, although its structure is not well defined (Crook and Hopkins, 1938). Recently, conclusive evidence for enzymes that catalyze the reduction of DHA by GSH in various animal tissues was obtained (Hughes, 1964; Grimble and Hughes, 1967; Wells, *et al.*, 1990). The reduction of DHA to ascorbic acid by cellular GSH is supported by recent work with intact animals although further work is necessary to detect whether the reaction is enzymatic or nonenzymatic (Meister, 1992). Interestingly, a glutaredoxin (thioltransferase) homolog encoded by vaccinia virus had dehydroascorbate reductase activity (Ahn and Moss, 1992). The existence of a functional DHA reductase encoded by a virus that infects eukaryotic cells suggests that the virus may benefit from the active GSH-dependent recycling of ascorbic acid to counteract the normal oxidative stress that it might otherwise encounter. Zilva (1927) first detected that dehydroascorbic acid was able to retain the physiological function of the natural vitamin. In 1928, Szent-Györgyi showed the recovery of the "reducing substance" from its oxidized state using glutathione as the reducing reagent. The mechanism of the reduction of dehydroascorbic acid and whether a free radical intermediate is required, is not understood.

There is good evidence for the participation of semidehydroascorbic acid free radical in the autoxidation of ascorbic acid (Bielski, 1982). Since the free radical is relatively nonreactive, it decays spontaneously by disproportionation to ascorbic acid and dehydroascorbic acid (rate = ca. $10^5\ M^{-1}\ s^{-1}$ at pH 7.0; Bielski, 1982):

$$2^{\bullet}AAH \rightleftharpoons AAH_2 + DHA$$

As an alternate pathway for ascorbic acid regeneration, reduction of the semidehydroascorbic acid free radical by NADH may be accomplished by the action of membrane-bound semidehydroascorbic reductases [EC 1.6.5.4; Kersten *et al.*, 1958; Schulze *et al.*, 1970]. Vitamin E and ascorbate interact chemically at lipid bilayer membrane cytosol interphases (Niki *et al.*, 1982). α-Tocopherol acts as the primary antioxidant free radical scavenger. The product, α-chromanoxyl free radical, is recycled to α-tocopherol by ascorbic acid, producing the ascorbic free radical. In this process, it is likely that NADH-semidehydroascorbate reductase can catalyze the regeneration of substantial amounts of ascorbic acid (Schneider and Staudinger, 1965). This activity was reported to be associated with mitochondria, microsomes, and plasma membranes (Goldenberg, 1980) and is the subject of other chapters in this series. In this review, emphasis is placed on the two-electron reduction of DHA by GSH.

PLANT DEHYDROASCORBATE REDUCTASE

The H_2O_2 produced by the dismutation of superoxide free radical in chloroplasts is removed via an ascorbate peroxidase-catalyzed pathway that produces DHA. Following the Szent-Györgyi (1928) suggestion that GSH could protect AAH_2 from oxidation, Pfankuch (1934) was the first to describe catalytic reduction of DHA by a thiol, in this case cysteine. In 1941, Crook characterized plant ascorbic acid oxidase activity and showed that it could be separated from the GSH-dependent DHA reductase-catalyzed reaction. DHA reductase was purified and characterized from spinach (Foyer and Halliwell, 1977; Hossain and Asada, 1984) and peas (Yamaguchi and Joslyn, 1952). The activity in spinach chloroplasts is associated with a protein of 23 kDA with apparent K_m values for GSH and DHA of 2.5 and 0.07 mM, respectively. The protein has a pI of 6.3 for the reduced form. Hossain and Asada (1984) tested various potential reductants including cysteine, NADH, NADPH, and thioredoxin *f* and *m*, but the reductase was strictly dependent on GSH.

IDENTIFICATION OF MAMMALIAN DEHYDROASCORBATE REDUCTASE

Early attempts to show the catalytic reduction of dehydroascorbic acid to ascorbic acid in mammalian tissue were unsuccessful (Borsook *et al.*, 1937; Schultze *et al.*, 1938). Reduction of dehydroascorbic acid, first observed by Szent-Györgyi in adrenal cortex, was attributed to chemical reduction of DHA by cellular GSH. These studies focused on the reversible oxidation–reduction of ascorbic acid to DHA and the irreversible conversion of DHA to

diketo-L-gulonic acid. From these studies, they concluded that DHA reductase does not occur in blood, but that there is rapid reduction of DHA in various tissues. The authors did not investigate whether this was or was not an enzyme-catalyzed reaction. However, in a study by Christine *et al.* (1956), evidence was obtained in human erythrocytes that an enzyme other than GSSG-reductase was involved in the reduction of DHA by GSH. In 1990, it was shown that two purified mammalian proteins, thioltransferase, also known as glutaredoxin, and protein disulfide isomerase, had dehydroascorbate reductase activity (Wells *et al.*, 1990). Although protein disulfide isomerase has two active center sequences that are shared with that of thioredoxin, the latter did not show DHA reductase activity. These results raise doubts as to the position of the active center in PDI for DHA-reductase activity. Using site-directed mutants of the active center for pig liver thioltransferase, Yang and Wells (1991) elucidated the mechanism of dithiol-disulfide exchange. These findings provided the basis for a proposed mechanism for dehydroascorbic acid reduction by glutathione discussed below.

Thioltransferase was recently isolated from human erythrocytes by Mieyal *et al.* (1991). The knowledge that thioltransferase possesses DHA reductase activity confirms the earlier suggestion by Christine *et al.* (1956) that there was an enzyme in human erythrocytes that could catalyze the reduction of DHA by GSH. As a result of our findings, we proposed a model for an electron transport chain that linked ascorbic acid with GSH and glutathione disulfide reduction via NADPH in the regeneration of ascorbic acid from dehydroascorbate (Fig. 1).

In other studies, especially of neutrophils, Bigley and Stankova (1974) found that these cells have a high capacity to regenerate ascorbate following its oxidation. Thus the reductase may function to protect cells

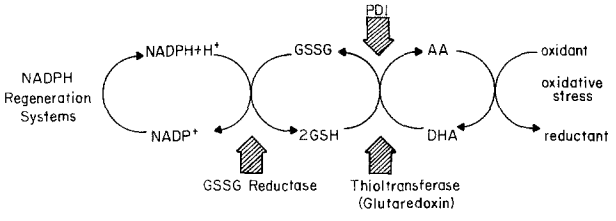

Fig. 1. Model of coupled electron transport pathway for the reduction of dehydroascorbic acid (DHA) via GSH-dependent systems involving either thioltransferase (glutaredoxin) or protein disulfide isomerase (PDI) (arrows).

from intoxication by the oxidants produced during phagocytosis. Bigley *et al.* (1981) measured the DHA reductase in crude cytosol extracts of human cells, finding that the K_m, for DHA was 1.3 mM and that for GSH was 3.8 mM for neutrophil and lymphocyte extracts. They also discovered that rates of DHA uptake by human neutrophils, monocytes, lymphocytes, and cultured fibroblasts were proportional to cytosol DHA reductase activities. Washko *et al.* (1993) reported on ascorbic acid levels of human neutrophils that increase during cell activation. When the external concentration of ascorbic acid was physiological, the internal concentration was as high as 14 mM. Based on the kinetic data, external ascorbic acid was oxidized to dehydroascorbic acid that is preferentially and rapidly transported across the plasma membrane. The mechanism of the DHA reduction in the neutrophil is unknown, although if it is shown that the recycling is glutathione dependent, thioltransferase or PDI are primary candidates for reductase activity. It is conceivable that alternative pathways exist for the regeneration of ascorbic acid from dehydroascorbic acid that do not depend on glutathione or membrane-associated NADH-dependent semidehydroascorbate reduction.

EVIDENCE THAT GSH IS ASSOCIATED WITH ASCORBIC ACID RECYCLING *IN VIVO*

Recently, Coassin *et al.* (1991) attempted to quantify in pig tissues the relative involvement of the two-electron, i.e., GSH-dehydroascorbate reductase (EC 1.8.5.1), and the one-electron, i.e., NADH-semidehydroascorbate reductase (EC 1.6.5.4), pathways in the regeneration of ascorbate from its various oxidation products. Based on enzyme assay of crude cellular fractions *in vitro*, and on reported K_m values for DHA in the mM range (200–260 μM was reported for porcine liver thioltransferase; Wells *et al.*, 1990; Wells *et al.*, 1994), the authors concluded that very likely DHA reductase activity does not play any relevant role in recycling ascorbate undergoing oxidation. This view does not accommodate the necessity to recycle intracellular dehydroascorbate generated by chemical disproportionation of semidehydroascorbate free radical. Similarly, dehydroascorbate generated by extracellular oxidation of ascorbate and actively transported by a sodium-dependent transporter (Washko *et al.*, 1993) will be regenerated by a two-electron or a one-electron

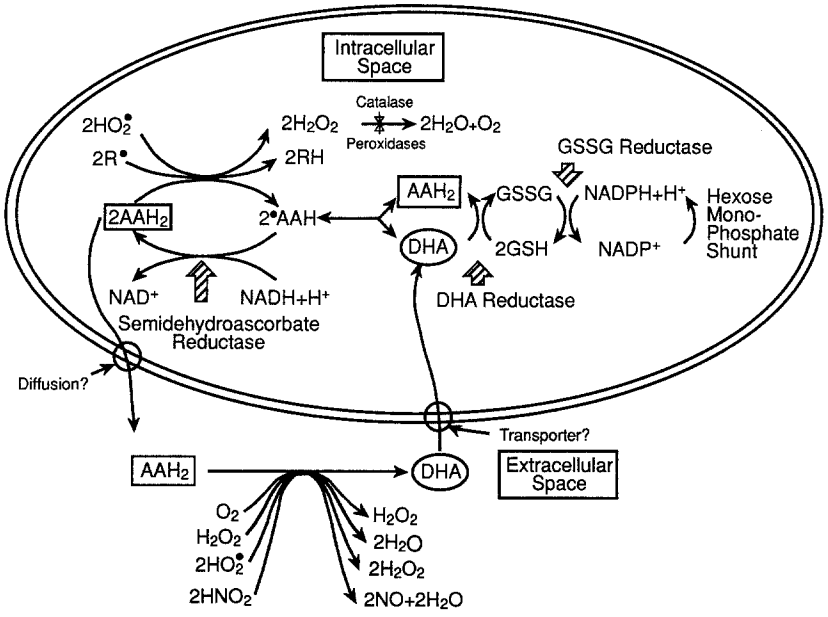

Fig. 2. Oxidation: reduction interrelationships of ascorbic acid (AAH_2), dehydroascorbic acid (DHA), and semidehydroascorbic acid ($^{\bullet}AAH$) in a model cell. Intracellular AAH_2 reacts with free radicals ($R^{\bullet}$), superoxide free radical ($HO_2^{\bullet}$), or other oxidants not shown, e.g., $^{\bullet}OH$ or H_2O_2. Single-electron oxidation leads to $^{\bullet}AAH$ which disproportionates into DHA and AAH_2. Alternatively, $^{\bullet}AAH$ is reduced back to AAH_2 by NADH via semidehydroascorbic acid reductase. Intracellular DHA may be recycled back to AAH_2 directly by DHA reductase and the GSH-linked electron transport pathway. Extracellular AAH_2 diffusing from cells or transported in plasma is oxidized by numerous oxidants eventually to DHA. The DHA is preferentially (compared with ascorbic acid) transported across the plasma membrane by a proposed DHA transporter (Washko *et al.*, 1993). The extracellularly produced DHA enters the intracellular pool where it may recycle to AAH_2 by a one- or two-electron pathway.

reduction (Fig. 2). Following the transport of extracellular DHA, intracellular DHA combined with intracellular ascorbic acid can produce low concentrations of semidehydroascorbic acid free radical. The equilibrium constant for this reaction at pH 6.4 and 25°C is 5×10^{-9} (Foerster *et al.*, 1965). The apparent K_m for semihydroascorbic acid in the recycling of the free radical intermediate to ascorbic acid is 4×10^{-6} M (Lumper *et al.*, 1967).

Support for the involvement of GSH in the regeneration of ascorbic acid *in vivo* comes from the extensive studies of Meister's laboratory (Meister, 1992). It was known for some time that administration of dehydroascorbate can prevent scurvy in γ-gulano lactone oxidase-deficient species, clearly showing the reduction of dehydroascorbate to ascorbic acid *in vivo*. An animal model was developed to test whether glutathione deficiency, induced by the administration of L-buthionine-SR-sulfoximine, a highly selective γ-glutamylcysteine synthetase inhibitor (Griffith and Meister, 1979), would affect the level and ascorbate/dehydroascorbate ratio in susceptible tissues. In newborn rats, severe glutathione deficiency leads to serious tissue damage including the lung (lamellar body destruction, loss of surfactant; Mårtensson *et al.*, 1991), liver (focal degeneration; Mårtensson and Meister, 1991; Mårtensson *et al.*, 1991), brain (cerebral cortex mitochondrial swelling; Jain *et al.*, 1991), and lens (cataracts; Mårtensson *et al.*, 1989). This type of tissue damage is associated with mitochondrial swelling presumably induced by failure to intercept the significant levels of respiration-derived H_2O_2 by GSH-peroxidase or ascorbate regeneration pathways.

Disposal of harmful free radicals by ascorbic acid generates semidehydroascorbic acid. Besides chemical disproportionation, some semidehydroascorbic acid is recycled to ascorbic acid catalyzed by the NADH-semidehydroascorbate reductase of mitochondria, microsomes, and plasma membranes (Fig. 2). This

process leads to an increase in membrane resistance to lipid peroxidation. However, tissue damage induced by GSH deficiency argues that nonglutathione mechanisms of ascorbic acid recycling cannot totally protect tissues from prooxidant stress. Glutathione deficiency induced by BSO administration produced marked depletion of tissue (liver, kidney, lung, brain, eye) ascorbic acid. In addition, the decreased total ascorbate was much higher in percentage of dehydroascorbate than normal. These results clearly show the requirement of GSH for ascorbic acid recycling, *in vivo*. The daily administration of large doses of ascorbic acid (ca. 2 mmol/kg/day) to BSO-treated animals decreased mortality significantly. As expected, daily administration of equal doses of dehydroascorbate to BSO-treated animals failed to protect the animals from glutathione deficiency symptoms. One surprising effect of the simultaneous administration of ascorbic acid to BSO-treated newborn rats was the increased level of GSH in tissues, i.e., a sparing effect of ascorbic acid on GSH metabolism. The ability to synthesize ascorbic acid in the newborn rat is low compared with the adult animal. Thus, adult mice can survive otherwise damaging glutathione deficiency, because they can synthesize increased amounts of ascorbic acid. When adult mice are treated with BSO, the ascorbic acid level of the liver increases about 2-fold within 4 h. The level of ascorbic acid then decreases and the dehydroascorbic acid level increases, consistent with the need for GSH and a two-electron recycling pathway for ascorbic acid. Meister's group further studied the effects of supplementary administration of glutathione monoethyl ester to guinea pigs fed an ascorbic acid-deficient diet. Scorbutic guinea pigs died of scurvy within 21–24 days, whereas those guinea pigs given the utilizable glutathione derivative survived significantly longer (Han *et al.*, 1992). Thus, each of the two antioxidants appears to closely influence the synthesis of the other. A deficiency of either one will cause an increase in the level of the other when possible for the common good of the cell subjected to oxidation stress. Although there is now good evidence in cells for the regeneration of ascorbic acid by glutathione, less is known about the requirement of catalytic activity that can influence the rates of GSH-dependent ascorbic acid formation. Currently, the only known dehydroascorbate reductases in mammalian cells are thioltransferase, found in the cytoplasmic fraction, and protein disulfide isomerase, located in the endoplasmic reticulum.

PREVIOUS ATTEMPTS TO ISOLATE MAMMALIAN ENZYMES IN THE REDUCTION OF DEHYDROASCORBATE

Hughes (1964) and Grimble and Hughes (1967) provided clear evidence for catalytic activity in tissues other than blood cells. Crude extracts from guinea pig tissues showed GSH-dependent ability to catalyze the reduction of dehydroascorbate, and some properties, such as relative thermostability and ionic properties, were reported. Stomach was shown to have the highest DHA-reductase activity. The activity was sensitive to proteolysis and sulfhydryl reagents. A dehydroascorbic acid reductase from carp hepatopancreas and kidney and from rat liver had properties that resembled those of thioltransferase (Yamamoto *et al.*, 1977).

The presence of a comparatively high level of DHA-reductase in stomach is of special interest. Ascorbic acid concentrations in gastric secretions of various species including humans are several times higher than plasma (Dabrowski, 1990). Accumulated data demonstrate that a considerable amount of ascorbate is secreted in the reduced form into the digestive tract and absorbed predominantly in its oxidized form, dehydroascorbate. The quantity of ascorbic acid, equivalent to the daily requirement, is secreted into the digestive tract of scurvy-prone animals including humans. This suggests an important function for the vitamin in the stomach, especially with respect to its antioxidant properties in the prevention of nitrosation. Nitrite can react with dietary amines and amides to create N-nitroso compounds implicated in gastric carcinogenesis. Ascorbic acid reacts with nitrite, converting it to nitric oxide, and generating dehydroascorbate in the process. In other studies, it was reported that patients with chronic gastritis have significantly lower stomach ascorbic acid content, suggesting disruption of the stomach ascorbic acid recycling process in these patients. Ascorbic acid may play another vital role nutritionally by enhancing the reduction of ferric ions to ferrous ions, the preferred redox state of iron for absorption. Thus, stomach tissue like neutrophils have a special requirement for DHA transport followed by ascorbic acid regeneration. This process correlates well with the high level of dehydroascorbate reductase found by Grimble and Hughes (1967) and the high level of thioltransferase (glutaredoxin-dehydroascorbate reductase) found in pig stomach by Gan and Wells (1988).

Table I. Kinetic Parameters for Mammalian Dehydroascorbate Reductases[a]

Enzyme	k_{cat}[b] (min^{-1})	DHA		GSH	
		K_m(app.)[c] (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_m(app.) (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Thioltransferase[d]	374	0.26	2.4×10^4	3.4	1.8×10^3
Protein disulfide[e] isomerase	16	2.80	1.0×10^2	2.9	0.9×10^2

[a]The parameters were determined using either constant GSH (3.0 mM) or DHA (1.5 mM). Reactions were run spectrophotometrically in 0.5 ml at 30°C, 200 mM sodium phosphate, pH 6.85, and 1 mM EDTA at 265 nm. All data were corrected for nonenzymatic reactions between DHA and GSH.
[b]k_{cat} values were calculated by dividing the V_{max}(app.) by the molar concentrations of the two enzymes.
[c]K_m(app.) was calculated by nonlinear least-squares fit of the velocity vs substrate concentration data.
[d]Recombinant pig liver thioltransferase (Yang and Wells, 1991).
[e]Bovine liver protein disulfide isomerase isolated according to a modification of Hillson *et al.* (1984) and Wells *et al.* (1994).

Choi and Rose (1989) investigated the regeneration of ascorbic acid by rat colon. These workers obtained a crude ammonium sulfate precipitate fraction that, after dialysis, catalyzed the reduction of dehydroascorbate in a NADPH-dependent manner. The authors did not rule out the presence of glutathione disulfide reductase and nondialyzable protein-bound glutathione in the crude extracts. Pietronigro *et al.* (1985), studying reductive metabolism of ascorbic acid in the central nervous system, could not detect dehydroascorbate reductase activity using their assay conditions. Despite the work of Washko *et al.* (1993) and Bigley *et al.* (1981) that strongly suggests the occurrence of dehydroascorbate reductase in leukocytes, Stahl *et al.* (1985) also failed to detect dehydroascorbate reductase activity in human leukocytes. These discrepancies in detection of DHA reductase activity may be explained on the basis of potential problems inherent in the preparation of tissue samples for dehydroascorbate reductase assay. First, the homogenizing buffer should contain a strong serine protease inhibitor such as phenylmethylsulfonyl fluoride (Wells *et al.*, 1994). Second, prior to assay, the cytoplasmic fraction should be concentrated using a device such as the Centricon-3 from Amicon, Inc. This step not only concentrates the proteins for better spectrophotometric sensitivity, but also eliminates some interfering cytosolic low-molecular-weight nucleotides or nucleosides that may otherwise contribute to an unmanageable high blank at 265 nm.

KINETIC STUDIES OF KNOWN MAMMALIAN DEHYDROASCORBATE REDUCTASES

Homogeneous recombinant pig thioltransferase has been further studied kinetically and compared with that of homogeneous bovine liver protein disulfide isomerase (Wells *et al.*, 1994). The kinetic parameters (Table I) show that thioltransferase is a moderately efficient dehydroascorbate reductase with k_{cat}/K_m of $1.3 \times 10^4\,M^{-1}\,s^{-1}$ for DHA. Protein disulfide isomerase is far less efficient ($0.95 \times 10^2\,M^{-1}\,s^{-1}$), but may make a significant contribution to overall cellular ascorbic acid recycling since PDI is estimated to be 0.4% of the total rat liver protein (Ohba *et al.*, 1981). We propose an alternative function for PDI in protein disulfide formation discussed below.

PROPOSED CATALYTIC MECHANISM FOR THIOLTRANSFERASE AS A DEHYDROASCORBATE REDUCTASE

A catalytic mechanism has been proposed for the interaction of thioltransferase or PDI in the reaction between DHA and GSH (Yang and Wells, 1991a, b; Wells *et al.*, 1993; Fig. 3). For pig liver thioltransferase, the nucleophilic Cys22 leads to a thiohemiketal intermediate. In the native enzyme, a cysteine at position 25 would displace the ascorbic acid, forming an intramolecular disulfide bond. Sequential reactions with GSH would regenerate the reduced enzyme and produce glutathione disulfide. Finally, GSH would be regenerated via the glutathione disulfide reductase system with electrons from NADPH. In the mechanism shown for the C25S mutant, GSH attacks the thiohemiketal intermediate, releasing ascorbic acid and forming a mixed disulfide between enzyme and glutathione. The enzyme is regenerated to the dithiol and glutathione disulfide by interaction with a second molecule of GSH. Using the mutants obtained previously for analysis of the thioltransferase

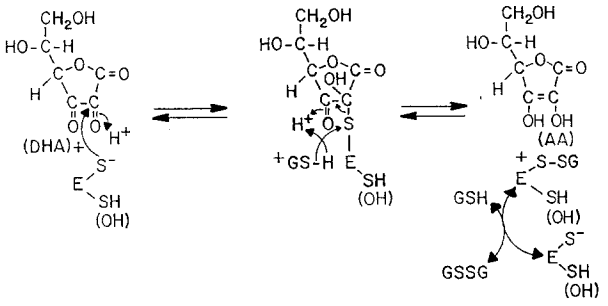

Fig. 3. Proposed scheme for the DHA reductase mechanism of thioltransferase. Reaction of the nucleophilic C22 leads to a thiohemiketal intermediate. The reduced product, ascorbic acid, is displaced by glutathione in the active mutant C25S to form a mixed disulfide that is further displaced by GSH to form GSSG and reduced enzyme. Alternatively, and not shown, an intramolecular disulfide pathway can displace the reduction product, ascorbic acid, followed by exchange with 2 moles of GSH.

mechanism, it will be possible to test the proposed mechanism in the future.

POSSIBLE ROLE OF DEHYDROASCORBIC ACID AND PDI IN PROTEIN DISULFIDE FORMATION

Most investigators agree that PDI is significantly involved in packaging or folding of the secretory proteins rich in disulfide bonds (Freedman, 1989). However, there is little concurrence on what the immediate oxidant is that drives the net synthesis of protein disulfide bonds. Cystamine (Poulsen and Ziegler, 1977) and glutathione disulfide (Creighton, 1984; Hwang *et al.*, 1992) have been proposed, but there is some good evidence that dehydroascorbate may be the primary oxidant. Venetianer and Straub (1964, 1965) used dehydroascorbic acid to oxidize the thiols of reduced inactivated ribonuclease A, a reaction verified by Givol *et al.* (1964). This action resulted in the regeneration of catalytically active ribonuclease A when the system also contained what is now known as PDI. The reaction is analogous to the chemical reduction of dehydroascorbate to ascorbic acid ($E_0' = +0.058$ V) by glutathione ($E_0' = -0.33$ V) and is thermodynamically feasible.

If dehydroascorbate is an essential oxidant in protein disulfide bond formation, it follows that animals with vitamin C deficiency would show symptoms of protein disulfide insufficiency. Indeed, old observations point to this possibility. Sigal and King (1936) reported that during development of scurvy in ascorbic acid-deficient guinea pigs, a corresponding rise in fasting blood sugar and a lowered glucose tolerance occurred. This observation was confirmed in a series of studies by Banerjee (1943, 1944). He used a crude hypoglycemia bioassay in rabbits to quantitate the insulin content of pancreases from scorbutic and normal guinea pigs. Banerjee concluded that the insulin content of the scorbutic guinea pig was diminished to about 12.5% of the normal guinea pig. We are currently using the scorbutic guinea pig as a model to investigate the potential role of ascorbic acid, converted to dehydroascorbic acid, to serve as oxidant in the biosynthesis of insulin and other selected disulfide proteins. A role for ascorbate and dehydroascorbate in this process requires an *in situ* oxidation of ascorbic acid to dehydroascorbate at the endoplasmic reticulum membrane surface. Robust ascorbic acid oxidase activity, usually measured by an oxygen electrode (Sun *et al.*, 1984), is known to occur in microsomes, but is poorly characterized. From kinetic data, Yamazaki and Piette (1961) concluded that a free radical mechanism is the main pathway involved in ascorbic acid oxidation catalyzed by plant ascorbate oxidase. Assuming some dehydroascorbic acid will result from disproportionation, it may then carry out the protein disulfide bond formation catalyzed by PDI, returning to the cytosol as ascorbic acid in the process. A scheme for this proposal is shown in Fig. 4.

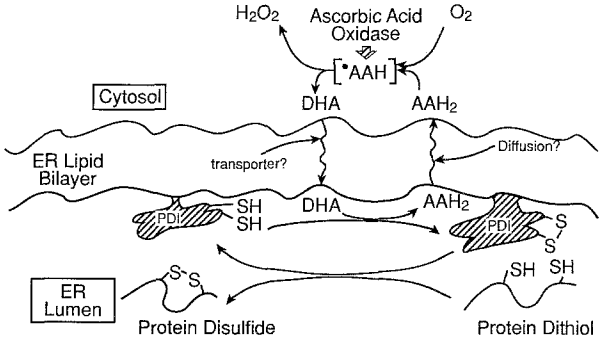

Fig. 4. Model for the proposed role of dehydroascorbate (DHA) in the protein disulfide isomerase (PDI) catalyzed formation of protein disulfides. Cytosolic AAH_2 is oxidized via •AAH to DHA by an ascorbic acid oxidase activity. DHA is transported across the ER lipid bilayer where it oxidizes the active center dithiols of PDI, regenerating AAH_2. Oxidized PDI reacts with nascent polypeptide chains destined for native protein disulfides, catalytically regenerating PDI.

FUTURE STUDIES TO ASSESS THE SIGNIFICANCE OF THIOLTRANSFERASE AND PDI IN ASCORBIC ACID METABOLISM

In unpublished studies, the authors have observed a correlation between elevated thioltransferase activity and the resistance of human MCF-7 breast tumor cells to Adriamycin (doxorubicin), a member of the quinone-semiquinone cycling drugs that induce elevated superoxide free radical cytotoxicity. When ascorbic acid is supplemented in the culture media as L-ascorbic acid-2-phosphate, the resistance of the drug resistant cells to Adriamycin is enhanced. Sensitive cells, which have no immunologically detectable thioltransferase, were not affected by supplementation with ascorbic acid 2-phosphate. Recently, the cDNA for human thioltransferase has been cloned in the author's laboratory (Meyer and Wells, unpublished data), thus providing the means to evaluate the effect of transfected and expressed thioltransferase (dehydroascorbate reductase) on cellular resistance to Adriamycin and other chemotherapeutic drugs. Another approach to investigating the functions of thioltransferase and its DHA reductase activity will be to transfect antisense cDNA for thioltransferase into human cells to evaluate the consequences of depleting the constitutive thioltransferase on ascorbic acid recycling and metabolism.

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REFERENCES

Ahn, B.-Y., and Moss, B. (1992). *Proc. Natl. Acad. Sci. USA* **139,** 7060–7064.
Banerjee, S. (1943). *Ann. Biochem. Exp. Med.* **III,** 157–164.
Banerjee, S. (1944). *Ann. Biochem. Exp. Med.* **IV,** 33–36.
Bielski, B. H. J. (1982). In *Ascorbic Acid: Chemistry, Metabolism, and Uses* (Seib, P. A., and Tolbert, B. M., eds.), Advances in Chemistry Series 200 (Comstock, M. J., ed.), American Chemical Soc., pp. 81–100.
Bigley, R. H., and Stankova, L. (1974). *J. Exp. Med.* **139,** 1084–1092.
Bigley, R. H., Riddle, M., Layman, D., and Stankova, L. (1981). *Biochim. Biophys. Acta* **659,** 15–29.
Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C. (1937). *J. Biol. Chem.* **117,** 237–279.
Choi, J.-L., and Rose, R. C. (1989). *Proc. Soc. Exp. Biol. Med.* **190,** 369–374.
Christine, L., Thomson, G., Iggo, B. Brownie, A. C., and Stewart, C. P. (1956). *Clin. Chim. Act* **1,** 557–569.
Coassin, M., Tomasi, A., Vannini, V., and Ursini, F. (1991). *Arch. Biochem. Biophys.* **290,** 456–462.
Creighton, T. E. (1984). *Methods Enzymol.* **107,** 3035–3329.
Crook, E. M. (1941). *Biochem. J.* **35,** 226–236.
Crook, E. M., and Hopkins, F. G. (1938). *Biochem. J.* **32,** 1356–1363.
Dabrowski, K. (1990). *Comp. Biochem. Physiol.* **95A,** 481–486.
Foerster, G. v., Weiss, W., and Staudinger, HJ. (1965). *Justus Liebig's Ann. Chem.* **690,** 166–169.
Foyer, C. H., and Halliwell, B. (1977). *Phytochemistry* **16,** 1347–1350.
Freedman, R. B. (1989). *Cell* **57,** 1069–1072.
Gan, Z-R., and Wells, W. W. (1989). *J. Biol. Chem.* **263,** 9050–9054.
Givol, D., Goldberger, R. F., and Anfinssen, C. B. (1964). *J. Biol. Chem.* **239,** PC3114–PC3116.
Goldenberg, H. (1980). *Biochem. Biophys. Res. Commun.* **94,** 721–726.
Griffith, O. W., and Meister, A. (1979). *J. Biol. Chem.* **254,** 7558–7560.
Grimble, R. F., and Hughes, R. E. (1967). *Experientia (Basel)* 23/5 362.
Han, J., Mårtensson, J., Meister, A., and Griffith, O. W. (1992). *FASEB J.* **6,** 5631.
Hillson, D. A., Lambert, N., and Freedman, R. B. (1984). *Methods Enzymol.* **107,** 281–293.
Hossain, M. A., and Asada, K. (1984). *Plant Cell Physiol.* **25,** 85–92.
Hughes, R. E. (1964). *Nature (London)* **203,** 1068–1069.
Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992). *Science* **257,** 1496–1502.
Jain, A., Mårtensson, J., Stole, E., Auld, P. A. M., and Meister, A. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 1913–1917.
Kersten, H., Kersten, W., and Staudinger, Hj. (1958). *Biochim. Biophys. Acta* **27,** 598–608.
Levine, M., Morita, K., and Pollard, H. (1985). *J. Biol. Chem.* **260,** 12942–12947.
Lindstedt, G., and Lindstedt, S. (1970). *J. Biol. Chem.* **245,** 4178–4186.
Lumper, vL., Schneider, W., and Staudinger, Hj. (1967). *Hoppe-Seyler's Z. Physiol. Chem.* **348,** 323–328.
Mårtensson, J., and Meister, A. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 4656–4660.
Mårtensson, J., Steinherz, R., Jain, A., and Meister, A. (1989). *Proc. Natl. Acad. Sci. USA* **86,** 8727–8731.
Mårtensson, J., Jain, A., Stole, E., Frayer, W., Auld, P. A. M., and Meister, A. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 9360–9364.
Meister, A. (1992). *Biochem. Pharmacol.* **44,** 1905–1915.
Mieyal, J. J., Stark, D. W., Gravina, S. A., Dothey, C., and Chung, J. S. (1991). *Biochemistry* **30,** 6088–6097.
Myllylä, R., Kuutti-Savolainen, E. R., and Kiririkko, K. I. (1978). *Biochem. Biophys. Res. Commun.* **83,** 441–448.
Niki, E., Tsuchiya, J., Tanimura, R., and Kamiya, Y. (1982). *Chem. Lett.*, 789–792.
Ohba, H., Harano, R., and Omura, T. (1981). *J. Biochem.* **89,** 889–900.
Ohmori, M., and Takagi, M. (1978). *Agric. Biol. Chem.* **42,** 173–174.
Pecherer, B. (1951). *J. Am. Chem. Soc.* **73,** 3827–3830.
Pfankuch, E. (1934). *Naturwissenschaften* **22,** 821.
Pietronigro, D. D., Hovsepian, M., Demopoulos, H. B., and Flamm, E. S. (1985). *Brain Res.* **333,** 161–164.
Poulsen, L. L., and Ziegler, D. M. (1977). *Arch. Biochem. Biophys.* **183,** 562–570.

Puget, K., and Michaelson, A. M. (1974). *Biochimie* **56,** 1255–1267.
Schneider, W., and Staudinger, Hj. (1965). *Biochim. Biophys. Acta* **96,** 157–159.
Schultze, M. O., Statz, E., and King, C. G. (1938). *J. Biol. Chem.* **122,** 395–406.
Schulze, H.-U., Gallenkamp, H., and Staudinger, Hj. (1970). *Hoppe-Seyler's Z. Physiol. Chem.* **351,** 309–317.
Sigal, A., and King, C. G. (1936). *J. Biol. Chem.* **116,** 489–492.
Sjostrand, S. E. (1970). *Acta Physiol. Scand., Suppl.* **356,** 1–79.
Stahl, R. L., Liebes, L. F., and Silber, R. (1985). *Biochim. Biophys. Acta* **839,** 119–121.
Sun, I. L., Morre, D. J., Crane, F. L., Safranski, K., and Crose, E. M. (1984). *Biochim. Biophys. Acta* **797,** 266–275.
Szent Györgyi, A. (1928). *Biochem. J.* **22,** 1387–1409.
Tolbert, B. M., and Ward, J. B. (1982). In *Ascorbic Acid: Chemistry, Metabolism, and Uses* (Seib, P. A., and Tolbert, B. M., eds.), Advances in Chemistry Series 200 (Comstock, M. J., ed.), *Am. Chem. Soc.*, pp. 101–123.
Venetianer, P., and Straub, F. B. (1964). *Biochim. Biophys. Acta* **89,** 189–190.
Venetianer, P., and Straub, F. B. (1965). *Acta Physiol. Acad. Sci. Hung.* **27,** 303–315.
Washko, P. W., Wang, Y., and Levine, M. (1993). *J. Biol. Chem.* **268,** 15531–15535.
Wells, W. W., Xu, D. P., Yang, Y., and Rocque, P. A. (1990). *J. Biol. Chem.* **265,** 15361–15364.
Wells, W. W., Yang, Y., Deits, T. L., and Gan, Z-R. (1993). In *Advances in Enzymol. and Related Areas of Molecular Biology* (Meister, A., ed.), **66,** 149–201.
Wells, W. W., Xu, D. P., and Washburn, M. P. (1994). *Methods Enzymol., Biothiols*, in press.
Yamaguchi, M., and Joslyn, M. A. (1952). *Arch. Biochem. Biophys.* **38,** 451–465.
Yamamoto, Y., Sato, M., and Ikeda, S. (1977). *Bull. Jpn. Soc. Sci. Fish.* **43,** 53–57.
Yamazaki, I., and Piette, L. H. (1961). *Biochim. Biophys. Acta* **50,** 62–69.
Yang, Y., and Wells, W. W. (1991a). *J. Biol. Chem.* **266,** 12759–12765.
Yang, Y., and Wells, W. W. (1991b). *J. Biol. Chem.* **266,** 12766–12771.
Zilva, S. S. (1927). *Biochem. J.* **21,** 689–697.