

Protein-disulfide Isomerase- and Protein Thiol-dependent Dehydroascorbate Reduction and Ascorbate Accumulation in the Lumen of the Endoplasmic Reticulum*

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The transport and intraluminal reduction of dehydroascorbate was investigated in microsomal vesicles from various tissues. The highest rates of transport and intraluminal isotope accumulation (using radiolabeled compound and a rapid filtration technique) were found in hepatic microsomes. These microsomes contain the highest amount of protein-disulfide isomerase, which is known to have a dehydroascorbate reductase activity. The steady-state level of intraluminal isotope accumulation was more than 2-fold higher in hepatic microsomes prepared from spontaneously diabetic BioBreeding/Worcester rats and was very low in fetal hepatic microsomes although the initial rate of transport was not changed. In these microsomes, the amount of protein-disulfide isomerase was similar, but the availability of protein thiols was different and correlated with dehydroascorbate uptake. The increased isotope accumulation was accompanied by a higher rate of dehydroascorbate reduction and increased protein thiol oxidation in microsomes from diabetic animals. The results suggest that both the activity of protein-disulfide isomerase and the availability of protein thiols as reducing equivalents can play a crucial role in the accumulation of ascorbate in the lumen of the endoplasmic reticulum. These findings also support the fact that dehydroascorbate can act as an oxidant in the protein-disulfide isomerase-catalyzed protein disulfide formation.

redox properties of these organelles are necessary for the formation and the maintenance of disulfide bonds in the secretory and plasma membrane proteins. Oxidizing conditions can be generated by the import of an oxidizing agent. It is apparently inconsistent with the above facts that these compartments contain ascorbate, a reducing compound, at high concentrations (3–5). The intraluminal accumulation of ascorbate can theoretically be explained by an active transport process or by its local generation from a membrane-permeable precursor. Whereas there are no data supporting the first possibility in the ER, the facilitated diffusion of dehydroascorbate (DHA), the oxidized form of ascorbate, has been described in rat liver microsomes. DHA uptake is presumably mediated by the glucose transporter T3 subunit of the glucose-6-phosphatase system (6). Local ascorbate oxidation and DHA formation have also been observed in microsomal vesicles (7). Therefore, ascorbate accumulation can be attributed to the intraluminal reduction of DHA taken up. However, enzyme(s) participating in the process and the source(s) of the reducing equivalents are unknown. Protein-disulfide isomerase (PDI), a major protein of the ER lumen, is known to have DHA reductase activity (8, 9). The aim of the present work was to explore the role of PDI in the intraluminal ascorbate accumulation in the ER. To this end, DHA transport, DHA reduction, and ascorbate accumulation were investigated in microsomes from various organs/cells having different PDI activities.

MATERIALS AND METHODS

Animals—The BioBreeding/Worcester (BB/Wor) male rats were provided by Møllegaard Breeding & Research Center A/S, Copenhagen, Denmark. 80-day-old BB/Wor healthy and insulin-implanted diabetic rats (180–200-g body weight) from the same colony were delivered by Charles River Ltd., Budapest, Hungary. Animals were fed *ad libitum* and housed in the rigidly controlled animal room of our laboratory. Blood glucose concentrations were tested each day until the exhaustion of the insulin implant. At that time, blood glucose levels in diabetic rats exceeded the 20 mM value; rats were kept for an additional 3 days in standard circumstances without any insulin supplementation and were then sacrificed. Serum glucose concentrations were 8.73 ± 0.68 and 28.24 ± 2.35 mM (means \pm S.E.; $n = 3$; $p < 0.01$) in control and diabetic rats, respectively. Male Wistar and Harlan Sprague-Dawley rats were obtained from Charles River Ltd., Budapest, Hungary.

Preparation of Microsomes—Liver microsomal vesicles were prepared from BB/Wor, Wistar, and Harlan Sprague-Dawley male rats (180–230-g body weight) as previously described (10). Fetal rat liver microsomes were prepared from 18-day-old fetuses (11). Nonhepatic microsomes were prepared by the same procedure from rat brain (12), rabbit skeletal muscle (13), fibroblasts, and J774 macrophages. Microsomes were resuspended at a concentration of 50–70 mg of protein/ml in Buffer A (20 mM MOPS containing 100 mM KCl, 20 mM NaCl, 1 mM MgCl₂, pH 7.2). The suspensions were frozen and maintained under liquid nitrogen until use. Intactness of microsomal vesicles was checked

The lumen of the endoplasmic reticulum (ER)¹ and of the vesicular structures of the whole secretory pathway is characterized by an oxidizing environment reflected in a high ratio of glutathione disulfide *versus* glutathione (1, 2). The suitable

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¹ The abbreviations used are: ER, endoplasmic reticulum; DHA, dehydroascorbate; PDI, protein-disulfide isomerase; BB/Wor, BioBreeding/Worcester; MOPS, 4-morpholinopropanesulfonic acid.

by measuring the latency of mannose-6-phosphatase (14) and *p*-nitrophenol UDP-glucuronosyltransferase (15) (in the case of liver microsomes) or by detection of the sustained light scattering signal due to the shrinking of vesicles upon the addition of the nonpermeant compound sucrose (in the case of nonhepatic and fetal liver microsomes) (14). To measure intravesicular sucrose spaces, microsomes were incubated overnight in Buffer A containing sucrose (1 mM) and its radiolabeled analogue (1 μ Ci/ml) at 4 °C and then samples were filtered and washed as described in the next paragraph.

Uptake Measurements—Microsomes (1 mg of protein/ml) were incubated in Buffer A containing the indicated amount of ascorbate or DHA and their radiolabeled analogues (1 μ Ci/ml) at 22 °C. At the indicated time intervals, samples (0.1 ml) were rapidly filtered through cellulose acetate/nitrate filter membranes (pore size 0.22 μ m), and filters were washed with 1 ml of Hepes buffer (20 mM; pH 7.2) containing 300 mM sucrose and 0.5 mM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid. The total radioactivity retained by filters was measured by liquid scintillation counting. In each experiment, the pore-forming agent alamethicin (0.1 mg/mg protein) (6) was added to parallel incubates to distinguish the intravesicular and bound radioactivity. The alamethicin-permeabilized microsomes were filtered and washed as above; the portion of released radioactivity was regarded as intravesicular.

Western Blot—Samples of liver microsomal fractions were sonicated and dissolved in 0.1 M Tris buffer, pH 8.0, containing 20 mM dithiothreitol and 1% Triton X-100. Microsomal proteins (15 μ g) were separated by electrophoresis on 9% sodium dodecyl sulfate polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF; Bio-Rad) membranes (overnight; 30 mA) (16). The membranes were blocked for 1 h with washing buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 2% bovine serum albumin and then probed with primary antibodies (SPA-890; StressGen) diluted in washing buffer for 1 h. After 3 washes with washing buffer containing 0.3% gelatin, horseradish peroxidase-conjugated secondary antibodies were added for 30 min. After 4 additional washes, the protein bands were detected using the enhanced chemiluminescence (ECL) technique (16) according to the manufacturer (Amersham Pharmacia Biotech).

PDI Activity—In a turbidimetric assay (17) the catalytic reduction and subsequent precipitation of reduced insulin β -chains were followed in 0.2 ml of 0.1 mM sodium phosphate buffer (pH 6.5) containing 2 mM dithiothreitol, 5 mM EDTA, 8–80 μ g of microsomal protein, and 80 μ M insulin at 37 °C. The aggregation was monitored at 650 nm using a Hitachi F-4500 spectrophotometer. PDI activity was also measured by a more sensitive fluorescent assay using the method of Heuck and Wolosiuk (18). Briefly, 4–20 μ g of microsomal protein was incubated in 0.2 ml of 0.1 mM sodium phosphate buffer (pH 7.4) containing 75 μ M dithiothreitol, 3 mM EDTA, and 0.7 μ M difluorescein-thiocarbonyl-insulin (a gift from A. P. Heuck and R. A. Wolosiuk) at 37 °C. Fluorescence was monitored using a Hitachi F-4500 spectrophotometer; excitation and emission wavelength were 495 and 520 nm, respectively.

DHA Reductase Activity—Microsomal DHA reductase activity was measured in Buffer A in the presence of 1 mM DHA and 2 mM glutathione as described (8), with the exception that ascorbate formation was detected according to Omaye *et al.* (19). The same experiments were also executed in conditions applied in the transport assay, *i.e.* in the absence of added glutathione.

Measurement of Metabolites—For the determination of protein thiol oxidation and ascorbate production intact or permeabilized microsomal vesicles (usually 1 mg of protein/ml) were incubated in Buffer A containing 1 mM DHA at 37 °C. For permeabilization microsomes were treated with alamethicin (0.1 mg/mg of protein). Incubations were terminated by the addition of 0.05 volume of 100% trichloroacetic acid. Ascorbate content was measured in trichloroacetic acid-soluble supernatants by the method of Omaye *et al.* (19), based on the reduction of Fe³⁺ by the oxidation of ascorbate and the subsequent determination of the Fe²⁺- α,α' -dipyridyl complex. Protein thiols were measured in the washed and resuspended pellets by the Ellman method (20). Protein concentrations were measured with Bio-Rad protein assay using bovine serum albumin as standard.

Chemicals—Ascorbate, alamethicin, diisothiocyanostilbene-2,2'-disulfonic acid, and glutathione were obtained from Sigma. L-[carboxyl-¹⁴C]Ascorbic acid (13.7 μ Ci/mmol) and [U-¹⁴C]sucrose (612 μ Ci/mmol) were from Amersham Pharmacia Biotech. DHA was produced by the bromine oxidation method according to Ref. 6. Cellulose acetate/nitrate filter membranes (pore size 0.22 μ m) were from Millipore. All other chemicals were of analytical grade.

TABLE I

DHA and ascorbate uptake in microsomes from various sources

Microsomes (1 mg/ml) were incubated in Buffer A in the presence of 1 mM ascorbate or DHA plus the radioactive tracer for 10 min at room temperature. After the incubation, 0.1 ml of sample was filtered as described under "Materials and Methods." Parallel samples were incubated in the presence of the pore-forming alamethicin; the radioactivity associated with these samples was regarded as background and was subtracted from the total radioactivity associated with the microsomes. The intravesicular sucrose space was measured by incubating microsomes overnight in Buffer A containing sucrose (1 mM) and its radiolabeled analogue at 4 °C and then samples were filtered as above. Data are means of four measurements. NM, not measured.

Source of microsomes	DHA uptake	Ascorbate uptake	Intravesicular sucrose space	Protein thiol content
	nmol/mg protein/10 min	nmol/mg protein/10 min	μ l/mg protein	nmol/mg protein
Fibroblasts	1.84	1.56	1.26	NM
Muscle	2.45	1.34	1.62	13.0
Brain	0.60	NM	NM	14.8
Macrophages	0.83	0.61	1.34	NM
Fetal rat liver	1.18	1.43	1.24	8.9
Rat liver	6.86	0.86	2.70	48.2

RESULTS

DHA and Ascorbate Transport in Microsomal Vesicles—Transport of DHA and of ascorbate was compared in microsomal vesicles of different origin. The highest DHA uptake was observed in adult rat liver microsomes, whereas in vesicles from other sources the uptake slightly exceeded or not exceed at all the level of the passive equilibrium (Table I). The time course of the uptake was also different; in nonhepatic microsomes the uptake reached a steady-state level in the first minute of incubation (data not shown), whereas in adult rat liver microsomes a continuous accumulation could be observed (Fig. 1). Ascorbate uptake was similar in all kinds of the investigated microsomal vesicles (Table I). Because the uptake can be influenced by the dimensions of the intravesicular space, the intravesicular sucrose space (which approaches the water space; see Ref. 11) was also measured. The intravesicular sucrose space of the various microsomes was similar (Table I) indicating that the difference in DHA transport was not because of the different size of the vesicles.

Effect of Diabetes on DHA Transport in Rat Liver Microsomal Vesicles—The time course of DHA uptake was investigated in microsomal vesicles prepared from the liver of diabetic and control BB/Wor and control Harlan Sprague-Dawley rats. DHA uptake exceeded the level of the passive equilibrium in all three different microsomes; no difference was observed in the DHA uptake of microsomes from nondiabetic rats. However, microsomes from diabetic BB/Wor animals showed a 3-fold higher intravesicular accumulation of radioactivity (Fig. 1.) than those from healthy control BB/Wor rats. The difference could be explained by the increased intravesicular metabolism of DHA rather than its faster uptake; the initial rate of uptake was similar in microsomes from diabetic and control rats (Fig. 1.). The time course and the steady-state level of ascorbate uptake were similar in all three experimental groups (data not shown).

Microsomal DHA Reduction—DHA reductase activity was measured in rat liver microsomes in conditions similar to those of the transport measurements, *i.e.* in the absence of added glutathione. Ascorbate formation was about 2-fold higher in diabetic microsomes (Table II). To clarify whether this enhanced DHA reduction is because of a more active reductase enzyme or an increased supply of reducing thiols, the experiments were repeated in the presence of glutathione. Because glutathione slowly enters microsomes (21), the vesicles were permeabilized with the pore-forming compound alamethicin. In

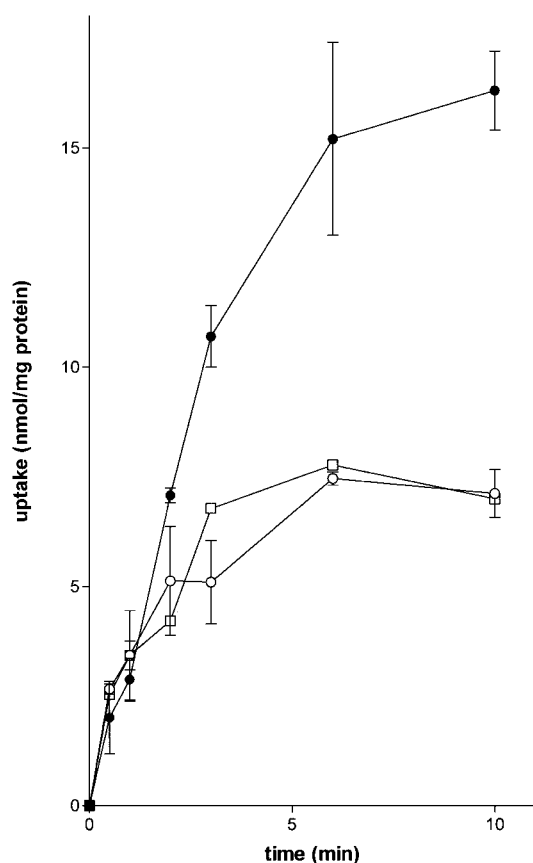


FIG. 1. Time course of DHA uptake in rat liver microsomal vesicles. Microsomes (1 mg of protein/ml) prepared from BB/Wor diabetic (●), BB/Wor control (○), or Harlan Sprague-Dawley (□) rats were incubated in the presence of 1 mM DHA plus the radioactive tracer at 22 °C. After the incubation, 0.1 ml of sample was filtered as described under "Materials and Methods." Parallel samples were incubated in the presence of the pore-forming alamethicin; the alamethicin-releasable portion of DHA associated with microsomes is shown. Data are mean \pm S.D. of three measurements or mean of two measurements.

TABLE II
Effect of diabetes on protein thiol content, DHA reductase activity, and DHA-dependent protein thiol oxidation in rat liver microsomal vesicles

Microsomes (1 mg of protein/ml) were incubated in Buffer A in the presence of 1 mM DHA. Ascorbate and protein thiol contents were detected at the beginning and after 30 min of incubation. DHA reductase activity was also measured in the presence of 2 mM glutathione for 10 min. Data are means \pm S.D.; $n = 3$.

	Microsomal protein thiol content	DHA reductase activity (with GSH)	DHA reductase activity (without GSH)	Protein thiol consumption
	nmol/mg protein	nmol/min/mg protein	nmol/min/mg protein	nmol/min/mg protein
BB control	52.9 \pm 1.2	4.98 \pm 1.71	0.228 \pm 0.030	0.183 \pm 0.036
BB diabetic	65.1 \pm 3.0	6.32 \pm 0.73	0.555 \pm 0.129	0.310 \pm 0.156

this case, the initial rate of ascorbate formation was only slightly higher in microsomes from diabetic animals (Table II). In the absence of GSH only the thiols of microsomal proteins can provide the reducing power for the reaction. Therefore, the increased DHA reduction could be because of a better supply of protein thiols. In accordance with this assumption, the initial protein thiol content was significantly higher in microsomes from diabetic rats, and a more pronounced oxidation of protein thiols could be observed in these microsomes upon DHA addition (Table II).

Microsomal PDI Level and Activity—Western blot analysis of

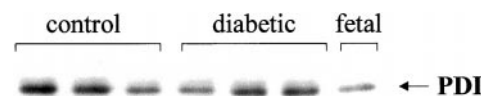


FIG. 2. Estimation of PDI level in the microsomal fraction of control, diabetic, and fetal rat liver. Western blotting of microsomal protein (15 μ g) was performed as described under "Materials and Methods."

liver microsomal samples from control and spontaneously diabetic rats did not show any increase in PDI protein content in diabetes. Fetal rat liver microsomes also contain similar amounts of PDI (Fig. 2). In accordance with the unchanged protein levels, neither the turbidimetric nor the fluorescent assay revealed a significant change of the oxidoreductase activity of PDI in these conditions (data not shown).

DISCUSSION

The transport of ascorbate and DHA through the membrane of the ER has been described in rat liver microsomal vesicles (6). The data presented here suggest that these transports are general phenomena in microsomal vesicles prepared from various tissues or cells. However, liver microsomes only display a significant accumulation of radiolabeled compounds upon DHA addition. This accumulation is likely because of the intravesicular reduction of DHA to ascorbate. Because microsomal preparations do not contain reducing equivalents in the form of NADPH or glutathione, the main source of electrons for the reduction of DHA must be protein thiols. The reaction between protein thiol groups and DHA can even occur nonenzymatically or can be catalyzed by PDI, an abundant enzyme of the (hepatic) ER (8, 9, 22–24). Our results suggest that PDI activity might have a decisive role in the intraluminal DHA reduction and ascorbate accumulation; the process was the most intensive where the highest PDI activity can be found, *i.e.* in liver microsomes (25, 26).

To further envisage the role of PDI, DHA transport was investigated in liver microsomes from spontaneously diabetic BB/Wor rats. An increased hepatic activity of PDI in experimental streptozotocin diabetes has been reported (27), whereas others found a decrease in the same conditions (28–30). We detected an increased accumulation of radiolabeled compounds upon 14 C DHA addition in microsomes from diabetic animals, although neither the level nor the activity of PDI was increased in these microsomes. Therefore, PDI activity *per se* cannot explain the differences in ascorbate accumulation in control and diabetic liver microsomes. The phenomenon can be explained by a different supply of reducing agents. In fact, the initial protein thiol level was higher, and the DHA-induced thiol oxidation was faster in microsomes from diabetic rats. It should be noted that streptozotocin diabetes also results in the elevation of protein thiols in liver microsomes.² On the other hand, fetal liver microsomes that contain low amounts of protein thiols exhibited moderate ascorbate accumulation despite their high PDI level. These findings suggest that at high PDI activity the availability of protein thiols can also determine the rate of intraluminal DHA reduction.

Ascorbate has been reported to promote protein disulfide formation in secretory proteins in the ER lumen (31). This process is hindered in diabetes; latent or intracellular scurvy appears because of the competition for the uptake between DHA and glucose at the plasma membrane (32), and metabolic hypoxia develops because of the excess of reducing power (33). Both factors can result in decreased protein thiol oxidation in the lumen of the hepatic ER. The increased production of DHA is verified in diabetes (34), and its accelerated uptake into the

² G. Nardai and P. Csermely, unpublished observation.

ER therefore can be regarded as a compensatory mechanism supporting protein disulfide formation in this pathological state.

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