Dehydroascorbate and Ascorbate Transport in Rat Liver Microsomal Vesicles*

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Ascorbate and dehydroascorbate transport was investigated in rat liver microsomal vesicles using radiolabeled compounds and a rapid filtration method. The uptake of both compounds was time- and temperature-dependent, and saturable. Ascorbate uptake did not reach complete equilibrium, it had low affinity and high capacity. Ascorbate influx could not be inhibited by glucose, dehydroascorbate, or glucose transport inhibitors (phloretin, cytochalasin B) but it was reduced by the anion transport inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid and by the alkylating agent N-ethylmaleimide. Ascorbate uptake could be stimulated by ferric iron and could be diminished by reducing agents (dithiothreitol, reduced glutathione). In contrast, dehydroascorbate uptake exceeded the level of passive equilibrium, it had high affinity and low capacity. Glucose *cis* inhibited and *trans* stimulated the uptake. Glucose transport inhibitors were also effective. The presence of intravesicular reducing compounds increased, while extravesicular reducing environment decreased dehydroascorbate influx. Our results suggest that dehydroascorbate transport is preferred in hepatic endoplasmic reticulum and it is mediated by a GLUT-type transporter. The intravesicular reduction of dehydroascorbate leads to the accumulation of ascorbate and contributes to the low intraluminal reduced/oxidized glutathione ratio.

Ascorbate producing and utilizing pathways are connected to the endomembrane system of the cell. The final enzymatic steps of ascorbate synthesis are located in the endoplasmic reticulum of hepatocytes or kidney cells; enzymes utilizing ascorbate (prolyl-3-hydroxylase, prolyl-4-hydroxylase, and lysyl hydroxylase) or its oxidized form dehydroascorbate (protein disulfide isomerase) are characteristic proteins of the lumen (1–3). Their presence in the lumen is necessary for the post-translational modification and folding of many proteins. Since ascorbate and dehydroascorbate are charged water-soluble compounds, transporter(s) should exist for their permeation through biological membranes. Such transporters have been thoroughly investigated in plasma membrane of different cells (4–9) and in chromaffin granula (10), but the transport of ascorbate and dehydroascorbate in microsomes has not been described in detail. The aim of the present study was to detect and characterize the activity of the possible ascorbate and/or dehydroascorbate transporter(s) in the endoplasmic reticulum.

**EXPERIMENTAL PROCEDURES**

**Preparation of Rat Liver Microsomes—**Microsomes were prepared from 24-h fasted male Sprague-Dawley rats (180–230 g) as reported (11). Microsomal fractions were resuspended in buffer A (100 mM KCl, 20 mM NaCl, 1 mM MgCl2, 20 mM MOPS, pH 7.2). The suspensions (60–80 mg of protein/ml) were rapidly frozen and maintained under liquid N2 until used. Intactness of microsomal vesicles was checked by measuring the latency of mannos-6-phosphatase (12) and p-nitrophenol UDP-glucuronosyltransferase activity (13), they were greater than 95% in all the preparations employed. Microsomal protein concentrations were determined by the biuret reaction using bovine serum albumin as standard. To measure microsomal intravesicular water space, microsomes were diluted (10 mg of protein/ml) in buffer A containing [3H]H2O (0.2 μCi/ml) or [3H]insulin (0.17 μCi/ml) and centrifuged (100,000 × g, 60 min), and the radioactivity associated with pellets was measured to enable calculation of extravesicular and intravesicular water spaces (14).

**Uptake Measurements—**Liver microsomes (1 mg of protein/ml) were incubated in buffer A containing the indicated amount of ascorbate, dehydroascorbate, or glucose and their radiolabeled analogues (1, 1, and 9 μCi/ml, respectively) 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 μm) and filters were washed with 1 ml of Hepes (20 mM) buffer (pH 7.2) containing 300 mM sucrose and 0.5 mM DIDS. The total radioactivity retained by filters was measured by liquid scintillation counting. In each experiment, the pore-forming agent a-lamcheticin (Ref. 15; 0.1 mg/ml protein) was added to parallel incubates to further evaluate the intravesicular and the bound radioactivity. The a-lamcheticin-permeabilized microsomes were filtered and washed as above; that portion of radioactivity so released was regarded as intravesicular (16).

Inhibitors used in the experiments (cytochalasin B, phloretin, DIDS, and N-ethylmaleimide) were added to the microsomes 30 min before the uptake measurement. The putative competitive inhibitors (ascorbate, dehydroascorbate, and glucose) and FeCl3 were added at the beginning of the uptake measurement. Loading of microsomes (10 mg of protein/ml) was accomplished by incubating them in the presence of the indicated compound for 30 min at 22 °C, then incubates were diluted 10-fold with buffer A containing ascorbate or dehydroascorbate.

**Light-scattering Measurements—**Osmotically induced changes in the size and shape of microsomal vesicles (17) after the addition of ascorbate or dehydroascorbate (12.5–12.5 mM) were monitored at 550 nm excitation and emission wavelengths by the light-scattering technique as described in detail in an earlier paper (18).

**Microsomal Metabolism of Ascorbate and Dehydroascorbate—**The ascorbic acid content (reduced and total) of microsomal incubates was measured by high performance liquid chromatography after specific sample preparation as described earlier (19, 20).

**Materials—**Ascorbate, a-methicin, DIDS, cytochalasin B, N-ethylmaleimide, phloretin, and D-[1-3H]glucose (15.5 Ci/mmol) were obtained from...
from Sigma. Dehydroascorbate was produced by the bromine oxidation method according to Ref. 21. L-[14C]Ascorbic acid (13.7 mCi/mmol) was from Amersham, Buckinghamshire, United Kingdom. Cel- lulose acetate/nitrate filter membranes were from Millipore. All other chemicals were of analytical grade.

RESULTS

The uptake of dehydroascorbate and ascorbate exhibited different kinetic characteristics with $V_{\text{max}}$ values of 3.1 and 37 nmol/min/mg protein and $K_m$ values of 0.7 and 45 mM, respectively (Fig. 1). The time course of the uptake processes showed that dehydroascorbate uptake exceeded the level of the passive equilibrium (3.5 nmol/mg protein; calculated from the intravesicular water space of microsomal vesicles: 3.5 $\mu$l/mg protein). The uptake of ascorbate reached only one-third of the level of equilibrium within 10 min (Fig. 2) and did not reach a complete equilibrium even after 1 h incubation (data not shown). Both uptake processes were temperature-dependent (Fig. 3).

The intravesicular accumulation of radioactivity upon dehydroascorbate addition in the absence of any source of energy in the incubation medium, indicated that microsomal metabolism of dehydroascorbate and ascorbate may have affected their transport. Indeed, a slow metabolism of ascorbate (4.0 ± 1.6 nmol/min/mg protein, mean ± S.D., $n = 4$; predominantly oxidation) and a more evident disappearance of dehydroascor-
ethylmaleimide inhibited the uptake of ascorbate more effectively than the anion transport inhibitor DIDS and the alkylating agent dehydroascorbate but not ascorbate uptake. On the other hand, dehydroascorbate transport. Phloretin and cytochalasin B inhibited ascorbate and dehydroascorbate transport through the plasma membrane, we checked the effect of GLUT inhibitors on ascorbate and dehydroascorbate transport. Phloretin and cytochalasin B inhibited dehydroascorbate but not ascorbate uptake. On the other hand, the anion transport inhibitor DIDS and the alkylation agent N-ethylmaleimide inhibited the uptake of ascorbate more effectively (Table I). Accordingly with the effect of GLUT inhibitors, glucose cis inhibited dehydroascorbate uptake (Fig. 4a), while from the trans side (i.e. in glucose-loaded vesicles) it was stimulatory (Fig. 2) suggesting that glucose and dehydroascorbate use the same microsomal transporter. Dehydroascorbate also cis inhibited the microsomal glucose uptake (Fig. 4c), supporting this assumption.

Ascorbate and dehydroascorbate did not influence the transport of each other (Fig. 4, a and b). Ascorbate inhibited glucose transport only at high concentrations presumably due to the shrinkage of vesicles (Fig. 4c), while glucose did not alter ascorbate influx (Fig. 4b).

Light-scattering experiments performed at high (12.5 mM) ligand concentration revealed that ascorbate was taken up by microsomes (for details see Ref. 16) and the permeabilization of vesicles by alamethicin resulted in a further influx. By contrast, dehydroascorbate rapidly entered the vesicles and after alamethicin addition only a minor further influx could be observed (Fig. 5).

**DISCUSSION**

Ascorbate and dehydroascorbate uptake in the endoplasmic reticulum, similarly to plasma membrane, appears to involve different transporters. Both processes are temperature, time, and microsomal protein dependent, saturable, and inhibitable, indicating that they are mediated by membrane proteins. The uptake of dehydroascorbate is preferred, it has higher affinity and for protein folding in the lumen (22, 23).

| Preincubation | Ascorbate uptake (nmol/min/mg protein) | Dehydroascorbate uptake (nmol/min/mg protein) |
|---------------|----------------------------------------|-----------------------------------------------|
| None          | 0.65 ± 0.15 (14)                       | 1.89 ± 0.12 (10)                                |
| 0.1 mM N-ethylmaleimide | 0.47 ± 0.01 (3)                       | 1.80 ± 0.36 (3)                                |
| 5 mM N-ethylmaleimide      | 0.06 ± 0.06 (3)                       | 0.92 ± 0.43 (3)                                |
| 0.25 mM DIDS              | 0.42 ± 0.04 (3)                       | 2.22 ± 0.18 (3)                                |
| 0.25 mM phloretin         | NM                                     | 1.46 ± 0.11 (3)                                |
| 0.25 mM cytochalasin B    | 0.59 ± 0.16 (6)                       | 0.48 ± 0.07 (5)                                |
| 2 mM dithiothreitol       | 0.70 (2)                               | 0.88 (2)                                       |
| 0.03 mM FeCl₃             | 1.74 (2)                               | NM                                            |

* NM, not measured.
whether this oxidation is mediated enzymatically (by a putative ascorbate oxidase) which could generate a high local dehydroascorbate concentration.

The results indicate that glucose and dehydroascorbate share the transporter not only in the plasma membrane, but also in the endoplasmic reticulum. GLUT1, GLUT2, and GLUT4 are efficient transporters of dehydroascorbate in the plasma membrane (4). These glucose transporters may also be present in the endomembranes due to vesicular transport and recycling (24); additionally, GLUT7, a microsomal glucose transporter (25, 26) can also mediate the transport of dehydroascorbate in the endoplasmic reticulum. Therefore, dehydroascorbate can be used as a surrogate or as a competitive inhibitor in the investigation of microsomal glucose transport, which is an important question in respect of the topology and mechanism of the glucose-6-phosphatase system (27). Moreover, high glucose levels (e.g. in diabetes) can efficiently inhibit the dehydroascorbate transport in the endoplasmic reticulum of hepatocyte and pancreatic β-cell. This effect worsens the intracellular shortage of ascorbate due to the decreased transport through the plasma membrane (6), may contribute to the inhibition of insulin secretion (28), and leads to latent scurvy (29) and elevation of plasma dehydroascorbate level (30).

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FIG. 4. Effect of ascorbate, dehydroascorbate, and glucose on the uptake of each other in rat liver microsomal vesicles. Transport measurements were performed at 1 mM concentration for 1 min. The alamethicin-releasable portion of dehydroascorbate (a), ascorbate (b), or glucose (c) associated with microsomes is shown. Ascorbate (●), dehydroascorbate (▲), and glucose (●) were added in the indicated concentrations. Data are mean ± S.D., n = 4–6, or mean of two measurements.

FIG. 5. Osmotically induced changes in light-scattering intensity of rat liver microsomal vesicles caused by dehydroascorbate or ascorbate. Light-scattering measurements were performed as described under "Experimental Procedures." Concentrated solutions (0.5 M) of dehydroascorbate (DHA) and ascorbate (AA) were added resulting in 12.5 mM final concentration. When indicated (A), alamethicin (0.1 mg/mg protein) was added. A typical set of experiments out of six is shown.
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