RESEARCH COMMUNICATION

Ascorbate oxidation is a prerequisite for its transport into rat liver microsomal vesicles

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Oxidation and uptake of ascorbate show similar time courses in rat liver microsomal vesicles: a rapid burst phase is followed by a slower process. Inhibitors of ascorbate oxidation (proadifen, econazole or quercetin) also effectively decreased the uptake of ascorbate. The results show that dehydroascorbate is the transport form of ascorbate at the membrane of the endoplasmic reticulum.

Key words: dehydroascorbate, endoplasmic recticulum, protein disulphide.

INTRODUCTION

The concentration of ascorbate is markedly different in the extracellular space, in the cytoplasm and in subcellular compartments of various cells, both in ascorbate-synthesizing and non-ascorbate-synthesizing species [1]. Ascorbate and its oxidized form, dehydroascorbate, are charged and are water-soluble compounds, and therefore their uneven distribution can be explained by the different rates and capacities of transport processes in the membrane. The ascorbate-transport systems of the plasma membrane are the best known; the intracellular accumulation of ascorbate is due to the Na^+-dependent co-transport of ascorbate and the facilitated diffusion and cytosolic reduction of dehydroascorbate [2–4]. Besides Na^+-dependent co-transport, it is questionable whether or not other forms of ascorbate transport exist. However, the intracellular and subcellular accumulation of ascorbate suggest that the facilitated diffusion of ascorbate can be absent, or minimally represented, in most membranes.

The lumen of the hepatic endoplasmic reticulum (ER) and of the vesicular structures of the secretory pathway is characterized by high (millimolar) ascorbate concentrations [5–7]. Ascorbate, in addition to its antioxidant properties, has another function: its accumulation creates a cofactor pool for several ascorbate-dependent intraluminal enzymes of the ER, such as prolyl hydroxylase and lysyl hydroxylase [1]. However, the mechanism of ascorbate accumulation is unknown. Although ascorbate transport seems to be less effective and can be inhibited by reducing agents, the facilitated diffusion of dehydroascorbate has been described in rat liver microsomes. It is presumably mediated by a glucose transporter, the T3 subunit of the glucose-6-phosphatase system [8]. These observations might suggest that ascorbate transport is dependent on its prior oxidation; that is, dehydroascorbate is the obligatory transport form in the ER. This mechanism is not unprecedented: superoxide anion produced by neutrophil granulocytes oxidizes ascorbate in the medium, dehydroascorbate is transported into the cells and it is reduced back to ascorbate in the cytosol. The whole process results in the intracellular accumulation of ascorbate [9]. Since ascorbate oxidation also takes place at the ER membrane [10,11], we considered the possibility that a similar mechanism might be responsible for ascorbate uptake and accumulation in this subcellular compartment. The aim of the present work, therefore, was to explore the role of ascorbate oxidation in intraluminal ascorbate accumulation in rat liver microsomal vesicles.

EXPERIMENTAL

Preparation of microsomes

Liver microsomal vesicles were prepared from Wistar male rats (250–300 g body weight) [12]. Microsomes were resuspended, frozen, and stored as described previously [8]. The intactness of microsomal vesicles was checked by measuring the latency of mannose-6-phosphatase and *p*-nitrophenol:UDP-glucuronosyltransferase [8].

Uptake measurements

Ascorbate transport was measured by a rapid-filtration method [8]. Inhibitors were added 10 min before the experiments. Ascorbate transport was also evaluated by the light-scattering method detailed in [8].

Ascorbate oxidation

For the determination of ascorbate oxidation, microsomal vesicles (1 mg of protein/ml) were incubated in the presence of 0.1 mM ascorbate at room temperature. Reactions were terminated and ascorbate contents were measured as described elsewhere [10]. Protein concentrations were measured with the Bio-Rad protein assay using BSA as standard.

Materials

Ascorbate, proadifen, econazole and quercetin were obtained from Sigma. l-[*carboxy*-^{14}C]Ascorbic acid (13.7 mCi/mmol) was from Amersham, Little Chalfont, Bucks., U.K. Cellulose acetate/nitrate filter membranes were from Millipore. All other chemicals were of analytical grade.

Abbreviation used: ER, endoplasmic reticulum.

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RESULTS

Ascorbate oxidation in rat liver microsomal vesicles

Rat liver microsomal vesicles were incubated in the presence of 0.1 mM ascorbate. On account of the instability and rapid re-reduction of dehydroascorbate, the oxidation of ascorbate was measured on the basis of decreases in its concentration. In the microsomal system the only likely metabolism of ascorbate is its oxidoreduction; the ascorbate consumption measured in our experiments represents a balance of oxidation and re-reduction [10]. An 'oxidative burst' was observed in the first 30 s of the incubation: approx. 15% of added ascorbate disappeared. This period was followed by a phase of slow, but continuous, ascorbate oxidation (Figure 1). In incubations carried out in the presence of inhibitors (proadifen, econazole or quercetin) the rate of ascorbate oxidation was significantly slower (Table 1).

Ascorbate transport into rat liver microsomal vesicles

Ascorbate (0.1 mM, plus radiolabelled tracer) was added to rat liver microsomal vesicles and its intravesicularly accumulated radioactivity was measured by a rapid-filtration method. Ascorbate uptake showed a biphasic behaviour – an initial fast phase (in the first minute of measurement) was followed by a second phase of slow transport (Figure 1). During the 10 min detection period, ascorbate uptake did not reach equilibrium; ascorbate occupied about 60% of the intravesicular water space (3.5 μl/mg of protein; see [8]). Addition of the inhibitors of ascorbate oxidation (proadifen, econazole or quercetin) inhibited ascorbate uptake (Table 1).

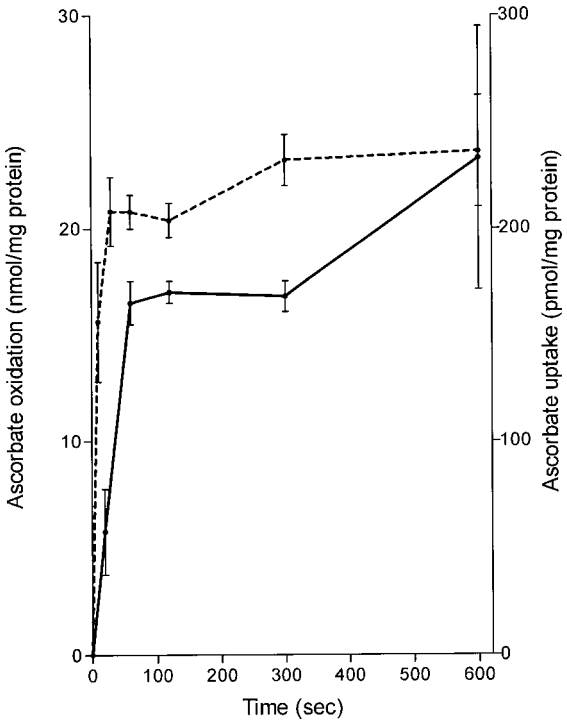

Figure 1 Time course of ascorbate oxidation and uptake in rat liver microsomal vesicles

Microsomes (1 mg of protein/ml) were incubated in the presence of 0.1 mM ascorbate at room temperature. At the indicated times the decrease in ascorbate concentration (broken line) was measured and ascorbate uptake (continuous line) was detected by a rapid-filtration method. Results are expressed as means ± S.D. for four to six experiments.

Table 1 Effect of the inhibitors of ascorbate oxidation on ascorbate uptake in rat liver microsomal vesicles

Microsomes (1 mg of protein/ml) were incubated in the presence of 0.1 mM ascorbate and the indicated inhibitors of ascorbate oxidation (0.1 mM each) at room temperature for 10 min. The decrease in ascorbate concentration was measured and ascorbate uptake was detected by a rapid-filtration method. The half-time of ascorbate transport was also calculated from the light-scattering traces shown in Figure 2. Results are expressed as means ± S.D. for four to six experiments. Abbreviation used: NM, not measured.

Addition	Ascorbate uptake or oxidation (nmol/10 min per mg of protein)		Ascorbate uptake half-time (s)
	Oxidation	Uptake	
None	23.6 ± 2.6	0.23 ± 0.06	5 ± 2
Econazole	4.5 ± 3.9	0.06 ± 0.01	29 ± 5
Proadifen	1.7 ± 1.4	0.05 ± 0.02	20 ± 5
Quercetin	10.4 ± 8.9	0.05 ± 0.01	NM

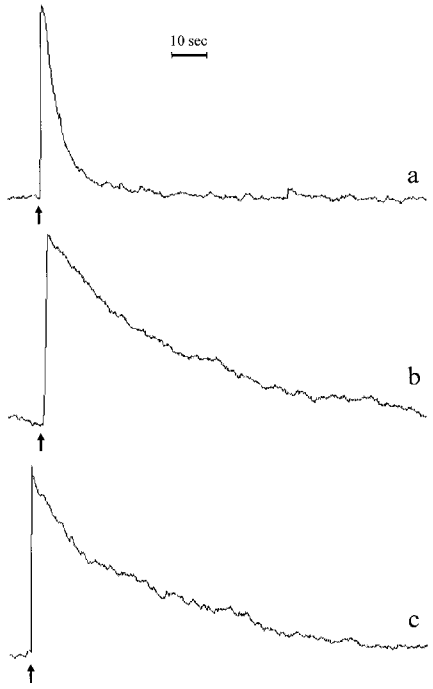

Figure 2 Ascorbate permeation in rat liver microsomes detected by a light-scattering technique

Microsomes (0.1 mg of protein/ml) were preincubated in the presence or absence of the inhibitors of ascorbate oxidation (trace a, control; trace b, 0.1 mM econazole; trace c, 0.1 mM proadifen) for 10 min. At the indicated time (arrow), 50 mM ascorbate was added and the osmotically induced changes in light-scattering were recorded. Representative traces from four experiments are shown.

Ascorbate uptake was also detected by a light-scattering technique. Ascorbate (50 mM) addition, in accordance with previous observations [8], resulted in the temporary shrinkage of rat liver microsomal vesicles, followed by a swelling phase, indicating the equalization of osmolarity on the two sides of the ER membrane due to ingress of osmolyte into the lumen. In the presence of econazole or proadifen the swelling phase became extended, showing a hindered permeation (Figure 2), with the half time of ingress increasing approx. 4–6-fold (Table 1).

These two compounds did not affect the permeation of other investigated molecules (glucose or sucrose; results not shown).

DISCUSSION

The transport of ascorbate and dehydroascorbate through the membrane of the ER has been described in rat liver microsomal vesicles [8]. The results presented here suggest that genuine ascorbate transport is negligible, or even absent, in microsomal vesicles, and the oxidation of ascorbate to dehydroascorbate is a prerequisite for the uptake. This assumption is supported by the following observations: (i) ascorbate oxidation and uptake display a similar time course in microsomes (Figure 1); (ii) inhibitors of oxidation also inhibit uptake (Table 1 and Figure 2); (iii) reducing agents decrease ascorbate uptake [8]; (iv) non-specific anion-transport inhibitors were only marginally effective in respect to ascorbate uptake [8].

The uptake did not reach equilibrium, even after 1 h incubation [8]; the concentration gradient for dehydroascorbate was therefore continuous. However, transport slowed down after the first minute, which can be attributed to the similar behaviour of ascorbate oxidation. A possible explanation for the slowing of ascorbate oxidation could be that the redox potential of the ascorbate–dehydroascorbate conjugate pair at this point approaches the redox potential value of the enzyme responsible for the oxidation.

The results indicate that ascorbate always arrives into the lumen of the ER in an oxidized form. Therefore, this transport process may be an important source of the oxidizing environment of the ER necessary for protein disulphide formation and protein folding. Dehydroascorbate can oxidize GSH or directly the protein thiol groups non-enzymically or by the mediation of protein disulphide-isomerase [15,16]. The reactions result in the reduction of dehydroascorbate and in the intraluminal entrapping of ascorbate.

The poor permeability of the ER membrane to ascorbate can explain the high ascorbate concentrations found in microsomal preparations or in the vesicular structures of the secretory pathway. The intraluminal entrapping of ascorbate creates a cofactor pool for ascorbate-dependent enzymes of the ER; moreover, it can be the source of ascorbate secretion by the hepatic exocytotic pathway, even in species unable to synthesize ascorbate [17].

In summary, the present results support the transport-based hypothesis for the generation of the oxidizing environment in the ER lumen. Ascorbate is able to promote protein thiol oxidation in microsomes; agents inhibiting microsomal ascorbate oxidation also inhibit its uptake and protein thiol oxidation. Altogether these findings strongly suggest that dehydroascorbate could be an oxidizing agent in the mechanism of protein disulphide formation.

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