Carrier mediated uptake of dehydroascorbate into higher plant plasma membrane vesicles shows trans-stimulation

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Received 9 September 1997; revised version received 14 November 1997

Abstract The activity of the ascorbate (Asc) carrier of purified *Phaseolus* plasma membranes is demonstrated to be highly stimulated when membrane vesicles are preloaded with Asc. Asc transport is inhibited by DTT but is not affected by glutathione or ferricyanide, indicating that dehydroascorbate (DHA) is the preferred species for uptake. Asc transport in the loaded vesicles showed saturable kinetics with an apparent affinity constant of 24 μM and maximal uptake rate of 94 pmol/mg/min. Addition of DHA stimulated the efflux of Asc molecules from the loaded vesicles. Together these results suggest the presence of an Asc/DHA exchange mechanism in higher plant plasma membranes.

Key words: Ascorbate; Plasma membrane; Ascorbate transport; Ascorbate/dehydroascorbate carrier; Counterflow; *Phaseolus*

1. Introduction

Apoplastic ascorbate (Asc) has been demonstrated to be involved in a number of different physiological phenomena including a role in cell growth and division and the protection of cells against oxidative stress (for review see [1]). However, the mechanisms controlling the extracellular Asc concentration and redox status are still poorly understood. For example, various studies have indicated that enzymes involved in the regeneration of Asc [1,2] could not be detected in the apoplast of plants [3–6]. Several authors have therefore suggested the presence of an Asc translocating system in the plant plasma membrane in order to maintain the apoplastic Asc concentration.

Uptake of labelled Asc into intact plant cells has been demonstrated in *Glycine* leaves and roots [7]. Asc uptake was also shown using protoplasts of *Hordeum* [8] and of *Pisum* [9]. Recently, experiments on purified plasma membrane vesicles of *Phaseolus* demonstrated the existence of a carrier mediated Asc transport system [10]. The *Phaseolus* Asc carrier was further shown to preferentially translocate oxidized dehydroascorbate (DHA) molecules from the apoplastic side of the membrane vesicles to the cytosolic side [11].

The actual mechanism of Asc transport through the membrane still remains a point of discussion. Using metabolic inhibitors Mozafar and Oertli [7] suggested an active process requiring metabolic energy. On the other hand, uptake in plant plasma membrane vesicles was not dependent on any chemical gradient tested so far and the mechanism of transport in in vitro conditions was described as facilitated diffusion [10]. Considering the major importance of Asc in plant cell physiology and the possible involvement of this carrier, transport of Asc into the purified plasma membrane vesicles was further characterized. The uptake process was studied using membrane vesicles containing a high concentration of intravesicular Asc. This condition more closely resembles the in vivo situation with a high ratio of cytosol to apoplastic Asc [9]. This paper provides the first experimental evidence suggesting an Asc/DHA exchange mechanism in the plasma membrane.

2. Materials and methods

Hypocotylar hooks of 5 day old bean seedlings (*Phaseolus vulgaris* L. var. Limburgse vroege) were harvested and collected on ice. The tissue (100 g fresh weight) was homogenized in 250 ml ice cold HEPES-KOH buffer (330 mM sucrose, 50 mM HEPES pH 7.5) supplemented with 3.8 mM cysteine, 0.5 mM PMSF and 0.6% PVP (insoluble) immediately before use. Plasma membrane vesicles were purified by aqueous two-phase partitioning as previously described [12]. After purification the vesicles were resuspended in 330 mM sucrose, 20 mM HEPES-KOH pH 7.5, stored on ice and used in uptake experiments within 3–4 h. The high purity of this fraction was previously demonstrated using marker enzymes [12].

Asc-loaded membrane vesicles were prepared as previously described by the addition of 10 mM Asc to the buffer immediately before tissue homogenization [12,13]. The pH of the homogenization buffer was readjusted with KOH.

Uptake of Asc by plasma membrane vesicles was measured in HEPES-KOH buffer (330 mM sucrose, 20 mM HEPES pH 7.5). Standard uptake experiments were carried out as described in Horemans et al. [10,11] by adding freshly prepared plasma membrane vesicles (40–60 μg protein) to 50 μM L-[^{14}C]ascorbic acid (Amersham, Gent, Belgium) to a final volume of 100 μl. After incubation the membrane vesicles were immediately diluted 50-fold with ice cold washing buffer (330 mM sucrose, 20 mM HEPES-KOH pH 7.5 and 10 mM non-labelled Asc), collected on a cellulose nitrate filter (0.45 μm, Sartorius, Göttingen, Germany) and rinsed by a further addition of 15 ml of washing buffer. The filters were dissolved in scintillation cocktail (Filter Count, Packard, Brussels, Belgium) and the radioactivity counted (liquid scintillation analyzer 1900 TR, Packard, Brussels, Belgium). For blank experiments samples were washed immediately after addition of the plasma membrane vesicles to the [^{14}C]Asc.

For the preparation of [^{14}C]Asc-loaded vesicles, a purified Asc-loaded membrane fraction was incubated with 50 μM [^{14}C]Asc. After 30 min the external [^{14}C]Asc was removed by diluting and repelleting the membranes (50 000 × *g*, 30 min).

3. Results

3.1. Preloading of membrane vesicles with Asc stimulates the uptake of ^{14}C-labelled Asc

Uptake of radioactively labelled Asc was determined in freshly purified Asc-loaded plasma membrane vesicles. The intravesicular Asc concentration is in the range of 500–1000 μM as analyzed by HPLC (data not shown). Despite the 10–20-fold higher concentration of Asc compared to the exter-

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PII S0014-5793(97)01534-2

Table 1
Uptake of [^{14}C]labelled Asc into Asc-loaded vesicles compared to non-loaded vesicles.

		Asc uptake ± S.E.	
		(pmol/mg)	(%)
Non-loaded		276 ± 12	(100)
	isosmotic wash	286 ± 38	(104)
	hypotonic wash	237 ± 100	(83)
Asc-loaded		879 ± 79	(318)***
	isosmotic wash	882 ± 160	(319)***
	hypotonic wash	240 ± 17	(87)

50 μg of membrane proteins were incubated together with 0.05 mM Asc for 20 min. Incubation conditions during the Asc uptake assay or washing treatment of the plasma membrane fraction are indicated. Statistical differences compared to the non-loaded vesicles are indicated: ***$P < 0.001$. Asc uptake is given as the mean of two separate isolations ± S.E. except for the control value of the non-loaded vesicles and the Asc-loaded where n is 25 or 12 respectively.

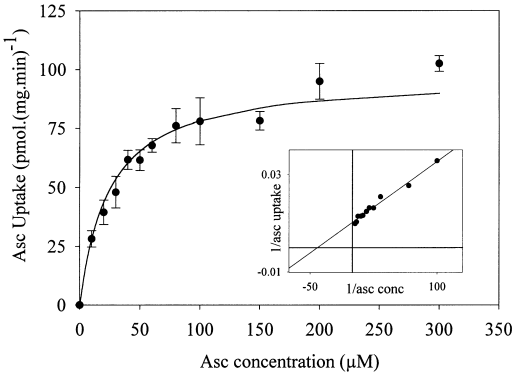

Fig. 2. Effect of [^{14}C]Asc concentration on Asc transport velocity into Asc-loaded vesicles. The curve described by the estimated K_m and V_{max} parameters (see text) is fitted onto the original data. Inset: Lineweaver-Burk plot with regression line ($r^2 = 0.98$).

nally added ^{14}C-Asc, a more than 3-fold increase in Asc uptake activity was observed when compared to non-loaded membrane vesicles ($P < 0.001$, $n = 12$, Table 1). In order to assess the role of the internal Asc, Asc-loaded vesicles were washed in hypotonic buffer conditions (no sucrose) prior to the uptake assay to remove the intravesicular Asc [14]. Following this treatment the transport activity was no longer different from the non-loaded vesicles (Table 1). Isosmotic washing of Asc-loaded vesicles did not affect the stimulated transport. These results strongly suggest a role of the intravesicular Asc in the stimulation of Asc uptake.

3.2. Redox status of the [^{14}C]Asc molecules transported by the carrier

Recently we demonstrated that the Asc carrier identified in purified plasma membranes preferentially translocates the oxidized DHA molecules. This was shown by adding oxidants and reductants to the external medium in order to control the Asc redox status during the uptake assay [11]. To determine the redox form in which Asc is transported into the Asc-loaded vesicles the effect of Asc reducing or oxidizing compounds was examined.

As summarized in Table 2 different concentrations of DTT resulted in significant decreases in the amount of Asc molecules transported into the Asc-loaded vesicles. At a concentration of 1 mM DTT only 129 pmol [^{14}C]Asc were transported after 20 min, representing an 82% inhibition of the uptake activity. Administering glutathione or ferricyanide, on the other hand, did not significantly affect the uptake of Asc.

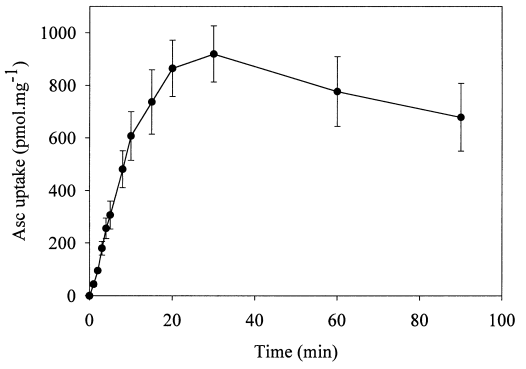

Fig. 1. [^{14}C]Asc uptake into Asc-loaded plasma membrane vesicles as a function of time. Asc (0.05 mM) was added to 50 μg membrane proteins. All values are means of two independent membrane isolations with S.E. values indicated.

3.3. Kinetic characterization of the [^{14}C]Asc uptake process into Asc-loaded vesicles

The uptake of labelled Asc molecules into Asc-loaded vesicles was followed as a function of time. The data presented in Fig. 1 show a curve which is linear ($r^2 = 0.96$) up to 20 min. A maximum of 900 pmol/mg Asc taken up is recorded after 30 min incubation. After this time, however, the level of labelled molecules taken up by the vesicles showed a tendency to decrease.

The uptake rate of [^{14}C]Asc into Asc-loaded vesicles was also recorded as a function of the concentration of labelled ligand (Fig. 2). To ensure that strictly initial uptake rates were measured, even at high [^{14}C]Asc concentrations, the incubation time was reduced to 6 min in these experiments. As indicated by the high linear correlation coefficient of the Lineweaver-Burk transformation ($r^2 = 0.98$, inset Fig. 2) Asc uptake in Asc-loaded vesicles apparently follows simple Michaelis-Menten kinetics. Uptake rate of Asc showed an apparent maximum of 97 pmol/mg/min (V_{max}) and an apparent affinity constant (K_m) of 24 μM.

Table 2
Asc uptake into Asc-loaded vesicels as affected by different compounds

		ASC uptake ± S.E.	
		(pmol/mg)	(%)
ASC-loaded		790 ± 76	(100)
+DTT	1 mM	129 ± 10	(18)**
	0.1 mM	314 ± 120	(43)*
	0.01 mM	620 ± 100	(86)
+Glutathione	1 mM	766 ± 40	(106)
	0.1 mM	763 ± 16	(106)
+FeCN	1 mM	843 ± 103	(112)
	0.1 mM	842 ± 108	(112)

Asc uptake was followed by addition of 0.05 mM [^{14}C]Asc to 50 μg of membrane proteins in the presence of various concentrations of the redox compounds DTT, glutathione and ferricyanide (FeCN). Uptake is given as the mean of two separate membrane isolations ± S.E. Statistical differences with respect to the control value are indicated: *$P < 0.05$ and **$P < 0.01$.

Table 3
Intravesicular [^{14}C] Asc content in [^{14}C]Asc-loaded vesicles as affected by external addition of non-labelled Asc or DHA

	[^{14}C]ASC concentration ± S.E.	
	(pmol/mg)	(%)
Buffer	510	(100)
+Asc+DTT	544 ± 40	(106)
+Asc	350 ± 17	(69)*
+DHA	346 ± 24	(68)*

The level of radioactivity in [^{14}C]Asc-loaded vesicles is indicated after incubating 50 μg of membrane proteins for 20 min. Different compounds were added to the incubation medium in the following concentrations: Asc 0.05 mM, DHA 0.05 mM, Asc+DTT 0.05 and 1 mM. Data are given as the mean of two separate membrane isolations ± S.E. Statistical differences with respect to the level in buffer are indicated: *$P < 0.05$.

3.4. Addition of DHA to [^{14}C]Asc-loaded vesicles stimulates Asc export

In animal cells, higher uptake rates of Asc carriers after preincubation with a non-labelled ligand has also been observed [15,16]. This effect is generally taken as an indication that the carrier operates through a counter-exchange mechanism. To test this hypothesis for the Asc carrier of bean plasma membranes we prepared [^{14}C]Asc containing membrane vesicles (see Section 2) and the efflux of labelled Asc from these vesicles after addition of Asc or DHA to the vesicle exterior was followed.

The level of labelled molecules retained in the vesicles was significantly lower ($P < 0.05$, $n = 2$) when the vesicles were incubated in the presence of Asc or DHA compared to incubation in buffer alone (Table 3). Addition of Asc+DTT (Table 3) or DTT alone (data not shown) did not affect the [^{14}C]Asc level. These experiments indicate that a significant amount of Asc was released from the vesicles when oxidized DHA molecules were added.

4. Discussion

Recently the activity of a carrier translocating Asc from the apoplast towards the cytoplasm of plant cells was demonstrated in a purified plasma membrane fraction of beans [10,11]. In order to address the possible physiological importance of this Asc uptake system, we attempted to determine Asc transport properties in membrane vesicles containing Asc and thus more closely resembling the in vivo situation. To our surprise about three times more labelled molecules were transported into Asc-loaded vesicles compared to the non-loaded ones. Washing of the vesicles in hypotonic conditions to remove the intravesicular Asc fully reversed this effect. These results suggested that the presence of Asc molecules inside the vesicles stimulated Asc uptake.

It is conceivable that the stimulating effect of Asc preloading was due to an antioxidative action of Asc resulting in a better protection of the membrane proteins during plasma membrane purification. Two observations argue against this explanation. First, Asc-loaded membrane vesicles were prepared after membrane purification by incubating them with non-labelled Asc prior to the [^{14}C]Asc uptake assay. These experiments also resulted in a highly stimulated uptake activity (up to two-fold, data not shown). Second, the standard medium used to homogenize the bean hook tissue already contains a high concentration of the antioxidant cysteine. In addition, supplementing the homogenization buffer with an additional 5 mM of the antioxidant DTT did not change the uptake in non-loaded vesicles (data not shown).

Taken together these results strongly suggest that the Asc carrier is stimulated by the presence of Asc inside the plasma membrane vesicles.

Stimulation of the uptake of a particular molecule by preincubation or preloading with a structural analogue has also been observed in other systems [15,17]. It is generally referred to as trans-stimulation or counterflow and taken as an indication for the presence of a mobile carrier operating by an exchange mechanism. Interestingly, transport of Asc in some animal cells was suggested to be driven by counterflow [15,16]. Since the data of this paper show trans-stimulation of Asc transport at the plasma membrane of plant cells we investigated the exchange mechanism using [^{14}C]Asc-loaded vesicles. Addition of DHA or Asc resulted in a significant release of [^{14}C]Asc from these vesicles. This effect was not observed when the added Asc was kept in a fully reduced state by addition of DTT supporting the specificity towards DHA. Although the redox status of the released molecules has not yet been determined, these results strongly support the hypothesis of an Asc/DHA exchange mechanism for Asc transport through the plasma membrane. These observations represent an important and new property of Asc transport in plant cells and point to similarities with transport systems in animal cells. As such this hypothesis supports new interpretations on the control of intra- and extracellular Asc concentrations in vivo. Considering the high level of reduced Asc molecules in the cytoplasm of plant cells [6,18], it seems likely that this counterflow operates as an in vivo exchange of intracellular Asc with apoplastic DHA. Although Asc/DHA exchange carriers have been demonstrated in animal tissues, based on the present data it is not yet possible to conclude whether the trans-stimulation observed in plant plasma membranes is mediated by a single carrier or by distinct proteins.

To investigate the possibility that Asc transport reactions in non-loaded vesicles [10,11] and Asc-loaded vesicles (this paper) were mediated by the same carrier, the kinetic properties were compared. In contrast to the results with non-loaded vesicles uptake of Asc into Asc-loaded vesicles followed simple Michaelis-Menten kinetics instead of a biphasic uptake activity. The reason for this difference is at present not clear. However, the apparent K_m value of Asc-loaded vesicles is close to K_{m1} (14 μM, [10]) of non-loaded vesicles and does not seem to be affected by the loading. The V_{max} value, on the other hand, was respectively two- and four-fold higher than those observed in non-loaded vesicles.

Similar to the results in non-loaded vesicles [11], a high inhibition of Asc transport is observed in the presence of DTT whereas glutathione and ferricyanide do not affect uptake activity. DTT has been shown to prevent external Asc from oxidation [11,19]. Addition of reduced glutathione (Table 2) and of the sulfhydryl reagents pCMBS and NEM [10] did not affect the uptake in Asc-loaded or non-loaded membrane vesicles. In addition supplementing the homogenization buffer with 5 mM of DTT did not affect Asc uptake activity (data not shown). It is therefore concluded that the inhibitory effect observed with DTT is not due to changes in the redox status of thiol groups present in membrane associated proteins but represents an effect on the redox status of Asc. The lower

uptake activity in the presence of DTT therefore clearly suggests that also in the Asc-loaded membrane vesicles DHA is the preferred substrate translocated from the outside to the inside.

Taken together, all characteristics of the Asc transport reaction demonstrated in Asc-loaded plasma membrane vesicles closely match the properties of Asc transport in non-loaded vesicles. We therefore suggest that the same carrier is involved in both processes.

Several physiological experiments suggested the possible presence of an Asc/DHA exchange between the plant cytoplasm and the apoplast. For example, Arrigoni [20] reported that the ability of *Vicia* roots to absorb Asc from the external medium depended on the high endogenous Asc level. Using *Spinacea* leaves it was demonstrated that the export of Asc from the cytosol into the apoplast depended on the presence of external DHA [21]. Moreover, during ozone fumigation of *Fagus* leaves the efflux of Asc apparently equals the uptake of apoplastic DHA [6]. The presence of an Asc/DHA exchange carrier in plant plasma membranes, as demonstrated in this paper, clearly provides a biochemical explanation for these observations. However, the operation of this counterflow carrier does not exclude the possibility of still another Asc transport system in the plasma membrane. The operation of different Asc transport systems in response to oxidative stress has been suggested by Luwe and coworkers [6,7].

Physiological experiments have demonstrated that the presence of reduced Asc in the apoplast protects cells against oxidative damage (for review see [1]). Takahama and coworkers showed, on the other hand, that reduced Asc strongly inhibits cross-linking of the cell wall and therefore directly affects cell growth processes [22]. These experiments clearly indicate that plant cells need to strictly control the redox status of Asc molecules in the cell wall in response to the needs of plant cell development as well as cell defence. Moreover, they suggest that the ratios of Asc/DHA in the cytosol and apoplast probably differ greatly. Further experimental work investigating the operation of ASC/DHA exchange in vitro as well as in vivo should help to understand the physiology and metabolism of Asc at the level of the plasma membrane.

Acknowledgements: H. Asard and N. Horemans are respectively a research associate and a doctoral worker at the Scientific Research Foundation - Flanders (FWO). P. Van Gestelen is acknowledged for the fruitful discussion of the results. This work is also supported by a grant of the FWO.

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