A plasma membrane-enriched fraction isolated from the coats of developing pea seeds contains $\text{H}^+$-symporters for amino acids and sucrose

A. de Jong$^1$ and A.C. Borstlap$^2$

Transport Physiology Research Group, Department of Plant Ecology and Evolutionary Biology, Utrecht University, Sorbonnelaan 16, NL-3584 CA Utrecht, The Netherlands

Received 22 May 2000; Accepted 24 May 2000

Abstract

Aqueous polymer two-phase partitioning was used to obtain a plasma membrane-enriched fraction from coats of developing pea (Pisum sativum L.) seeds in the filling stage. Uptake of amino acids and sucrose by vesicles from this fraction was determined after imposition of gradients of proton concentration ($\Delta$pH, inside alkaline) and electrical potential ($\Delta\psi$, inside negative) across the vesicle membrane. The uptake of sucrose and the amino acids L-valine, L-lysine, and L-glutamic acid was stimulated by the imposition of $\Delta$pH. The imposition of $\Delta\psi$, either in the presence or in the absence of $\Delta$pH, stimulated the uptake of L-valine and L-lysine, but had no detectable effect on the uptake of sucrose and L-glutamic acid. The proton-motive-force-driven uptake of all four substrates was abolished by the protonsophore carbonylcyanide m-chlorophenyl-hydrazone (CCCP). The results demonstrate the presence of $\text{H}^+$-symporters for sucrose and amino acids in pea seed coats. This is running counter to the previously reported finding that their uptake by isolated pea seed coats was insensitive to CCCP, and that the uptake of sucrose, L-valine, and L-glutamic acid displayed linear kinetics. Possible causes of this discrepancy will be discussed.

Key words: Amino acids, plasma membrane, proton symport, seed coat, sucrose.

Introduction

Sucrose and amino acids are generally the main organic constituents of phloem sap, and the most important nutrients for developing seeds. Specialized tissues, such as the seed coat parenchyma in seeds of legumes and other dicots and the nucellar strand in cereal grains are committed to release the phloem-imported nutrients into the apoplastic space from which they are taken up by the endosperm or tissues of the plant embryo (for reviews: Thorne, 1985; Fisher, 1995; Patrick, 1997). The release of solutes from cells implies their transport across the plasma membrane, and because the permeability of lipid bilayers to hydrophilic solutes like sugars and amino acids is extremely low (Brunner et al., 1980; Chakrabarti and Deamer, 1992), this passage is probably facilitated by some kind of transporter. Transporters dedicated to the release of sugars or amino acids from plant cells have not yet been identified.

Although the primary function of $\text{H}^+$-symporters for sugars and amino acids is to enable the plant cell to accumulate these solutes from the apoplastic space, a possible involvement in the release of their substrates from the cell cannot be dismissed a priori. The direction of the net flux of substrate through a $\text{H}^+$-symporter depends on the electrochemical gradients for protons and the substrate. In solute-releasing tissues the direction and magnitude of the gradients might be such that a net efflux of sugars and amino acids occurs by $\text{H}^+$-symporters. However, apoplastic concentrations of sugars and amino acids in developing seeds (Thorne, 1985) are probably...
much higher than is expected when their transport across the plasma membrane were under the strict control of tightly-coupling H⁺-symporters.

Conceptually it seems more reasonable that plant cells employ H⁺/antiporters to release assimilates into the apoplastic. This has been suggested for the delivery of sucrose by seed coats of *Vicia faba* and *Phaseolus vulgaris* (Fieuw and Patrick, 1993; Walker et al., 1995). This suggestion was based on the inhibition of the release by 100 μM CCCP and by lower H⁺-concentrations in the apoplast. On the other hand, the release of sucrose and amino acids from detached seed coats of *Pisum sativum* was not affected by 10 μM CCCP, nor by raising the pH from 5.5 to 7.5 (De Jong and Wolswinkel, 1995). The influx of sucrose and amino acids into isolated seed coats showed linear kinetics and was inhibited by PCMBs to the same extent as the release of the endogenous solutes. In addition, the permeability of the plasma membrane of seed coat parenchyma cells, as probed in uptake experiments, seems to be sufficient to release sucrose and amino acids at the required rates. On this basis it has been suggested that assimilates are released from seed coat parenchyma cells through proteinaceous, non-selective pores in their plasma membranes (De Jong et al., 1996, 1997). In a somewhat different experimental approach, Wang and Fisher had arrived at a similar conclusion for the release of sucrose from the nucellar strand in the developing wheat grain (Wang and Fisher, 1995).

A possible candidate for the facilitated release of sucrose from seed coat parenchyma cells is the sucrose-binding protein (SBP). This protein, originally isolated from cotyledons of developing soybean seeds (Ripp et al., 1988), shares structural and sequence similarity with seed storage proteins (Overvoorde et al., 1997). The protein is associated with the plasma membrane of seed coat parenchyma cells (Harrington et al., 1997), but also in mesophyll cells of young sink leaves, the companion cells in the phloem of mature leaves, and cells of developing cotyledons (Grimes et al., 1992). Apparently, SBP is expressed in cells that export sucrose as well as in cells that import sucrose. The expression of SBP in yeast cells led to the induction of a transport pathway for sucrose which displayed linear kinetics and was insensitive to protonophores (Overvoorde et al., 1996). Though this is highly suggestive of SBP acting itself as a transporter, the possibility that its expression induces an independent pathway, or activates an existing one, cannot be ruled out. The fact that SBP is a hydrophilic protein with only a single putative transmembrane segment is not in favour of its hypothetical function as a transporter. Moreover, SBP is a sucrose binding protein, whereas sucrose transport in the SBP-expressing yeast cells shows linear kinetics, indicating a very low affinity, if any, of the transporter for sucrose.

The availability of a method for the isolation of plasma membrane vesicles (Larsson et al., 1987) provides an opportunity to study transport processes at the membrane level. By choosing appropriate media for the isolation of the vesicles and the uptake experiments it should be possible, in principle, to demonstrate the activities of H⁺-symporters as well as of H⁺-antiporters. In this paper, the isolation of a plasma membrane-enriched fraction from pea seed coats and evidence for the presence of H⁺-symporters for sucrose and amino acids is presented. The H⁺-symport of sucrose and amino acids in plasma membrane vesicles isolated from cotyledons of developing pea seeds is reported elsewhere (De Jong and Borstlap, 2000).

### Materials and methods

#### Plant material

Pea (*Pisum sativum* L. cv. Marzia) seeds were obtained from Nunhems Zaden BV, Haelen, The Netherlands. Pea plants were grown in a growth chamber as described previously (De Jong and Wolswinkel, 1995) except that no flowers were removed from the plants. The cotyledons of the immature seeds used for the isolation of plasma membranes from the seed coats had a water content of 55–75%.

#### Isolation of a plasma membrane-enriched fraction

Plasma membranes were isolated from a microosomal fraction by aqueous two-phase partitioning (Larsson et al., 1987). The protocol used was that described for the isolation of plasma membranes from developing cotyledons (De Jong and Borstlap, 2000) with a few modifications. Seed coats (70–100 g FW) were homogenized in 200 ml of homogenization medium. The first centrifugation step (4200 g) in the isolation of the microsomal fraction was omitted, and the two-phase system contained 6.2% (w/w) dextran T500 (Pharmacia), 6.2% (w/w) polyethylene glycol 4000 and 5 mM KCl. After partitioning the two-phase systems were centrifuged for 3 min at 1500 g.

#### Assays for protein, marker enzymes and transport activity

Protein, activities of marker enzymes and uptake of ¹⁴C-labelled substrates by membrane vesicles were determined as described (De Jong and Borstlap, 2000).

### Results

#### Biochemical characterization of the plasma membrane fraction

The plasma membrane-enriched fraction was obtained by pooling the two upper phases, U₁ and U₂ which resulted from the partitioning of a microsomal fraction against three lower phases. About 4% of the protein in the microsomal fraction was recovered in U₁ + U₂ (Table 1). The plasma membrane fraction was almost colourless and the specific activity of cytochrome c oxidase in it was 45 times lower than that in the microsomal fraction. The
Table 1. Protein and activities of marker enzymes in the microsomal fraction, the first lower phase (L₁), and the upper phases (U₁ + U₂)

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Microsomal fraction</th>
<th>L₁</th>
<th>(U₁ + U₂)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase</td>
<td>17.5 ± 0.8</td>
<td>10.1 ± 1.7</td>
<td>0.74 ± 0.16</td>
<td>58 ± 4.2</td>
</tr>
<tr>
<td>Glucan synthase II</td>
<td>43 ± 7</td>
<td>46 ± 12</td>
<td>0.037 ± 0.005</td>
<td>107 ± 0.09</td>
</tr>
<tr>
<td>ATPase*</td>
<td>149</td>
<td>64</td>
<td>5.9</td>
<td>43 ± 4.0</td>
</tr>
<tr>
<td>Vanadate-inhibitable</td>
<td>9.1 ± 0.7</td>
<td>3.9 ± 0.6</td>
<td>1.1 ± 0.2</td>
<td>43 ± 12.1</td>
</tr>
<tr>
<td>Baflomycin-inhibitable</td>
<td>5.6 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>0.52 ± 0.18</td>
<td>45 ± 9.3</td>
</tr>
</tbody>
</table>

* Determined in the presence of molydate and azide.

Table 2. Specific activities (nmol mg⁻¹ protein min⁻¹) of marker enzymes in the microsomal fraction, the first lower phase (L₁), and the combined upper phases (U₁ + U₂)

<table>
<thead>
<tr>
<th></th>
<th>Microsomal fraction</th>
<th>L₁</th>
<th>(U₁ + U₂)</th>
<th>Enrichment in (U₁ + U₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase</td>
<td>2429 ± 322</td>
<td>4374 ± 686</td>
<td>54 ± 9</td>
<td>0.022</td>
</tr>
<tr>
<td>Glucan synthase GSII*</td>
<td>9.4</td>
<td>8.6</td>
<td>12.3</td>
<td>1.31</td>
</tr>
<tr>
<td>ATPase, total activity*</td>
<td>517 ± 19</td>
<td>386 ± 14</td>
<td>1469 ± 111</td>
<td>2.84</td>
</tr>
<tr>
<td>Vanadate-inhibitable</td>
<td>319 ± 12</td>
<td>251 ± 17</td>
<td>663 ± 181</td>
<td>2.08</td>
</tr>
<tr>
<td>Baflomycin-inhibitable</td>
<td>95 ± 20</td>
<td>75 ± 23</td>
<td>513 ± 167</td>
<td>5.40</td>
</tr>
<tr>
<td>Vanadate inhibition (%)</td>
<td>62 ± 1</td>
<td>65 ± 2</td>
<td>44 ± 10</td>
<td>1.82</td>
</tr>
<tr>
<td>Baflomycin inhibition (%)</td>
<td>19 ± 4</td>
<td>19 ± 5</td>
<td>34 ± 10</td>
<td>1.82</td>
</tr>
<tr>
<td>Latency (%)†</td>
<td>37 ± 1</td>
<td>27 ± 1</td>
<td>75 ± 4</td>
<td>1.82</td>
</tr>
</tbody>
</table>

* Single preparations.
† Determined in the presence of molydate and azide.
‡ Latency of the vanadate inhibitable ATPase activity; mean values for two isolations.

plasma membrane markers, vanadate-inhibitable ATPase and glucan synthase II were slightly enriched (2.0- and 1.3-fold, respectively) in the plasma membrane fraction, whereas the baflomycin-inhibitable ATPase was enriched about 5-fold (Table 2). Only 44% of the ATPase activity could be inhibited by vanadate, but as much as 34% was inhibited by baflomycin. These results indicate that membranes containing a V-type ATPase co-purified with the plasma membranes. About 75% of the vesicles in U₁ + U₂ were right-side-out, as shown by the latency of the vanadate-inhibitable ATPase activity (Table 2).

Uptake of amino acids

The uptake of a neutral (L-valine), a basic (L-lysine) and an acidic amino acid (L-glutamic acid) by plasma membrane vesicles was determined under four conditions: after the simultaneous imposition of ΔpH + Δψ, after imposition of ΔpH or Δψ alone, and when no gradients were present (Fig. 1). For all three amino acids some time-dependent uptake was measured in the absence of gradients. After the imposition of ΔpH + Δψ uptake of the amino acids was rapid during the first 2 min, and reached a plateau within 4 min of incubation which was approximately 5-fold higher than in the absence of the gradients. Similar results were obtained for the uptake of L-alanine, L-threonine and L-arginine (data not shown). The proton-motive-force-driven uptake of valine, lysine and glutamic acid was prevented when CCCP was included in the uptake medium (Fig. 2a).

Uptake of the three amino acids was notably different with respect to the stimulating effect of Δψ. The imposition of Δψ increased the ΔpH-dependent uptake of valine nearly 3-fold, and that of lysine approximately 6-fold, but did not affect the ΔpH-dependent uptake of glutamic acid. When the gradients were imposed alone the uptake of valine was more strongly stimulated by ΔpH than by Δψ, the reverse being true for the uptake of lysine (Fig. 1).

Accumulation of amino acids in response to the imposition of ΔpH + Δψ was calculated assuming an intravesicular volume of 5 μl mg⁻¹ protein. The neutral amino acids alanine, threonine and valine accumulated in the vesicles by factors of 30-50. Even higher accumulation ratios of 70-100 were found for the basic amino acids lysine and arginine, and the acidic amino acid glutamate (Fig. 3).
Fig. 1. Uptake of amino acids by vesicles of the plasma membrane-enriched fraction. Uptake was determined after the imposition of $\Delta \Delta \psi + \Delta \Delta \psi$ (○), $\Delta \Delta \psi$ (●), and in the absence of gradients (■). (a) Uptake of l-valine supplied at a concentration of 1.12 μM. Symbols represent the mean values ± SE of six (○) or three (●, ■) preparations. (b) Uptake of l-lysine supplied at a concentration of 0.85 μM. Symbols represent the mean values ± SE of two preparations. (c) Uptake of l-glutamic acid supplied at a concentration of 1.04 μM. Symbols represent the mean values ± SE of three (○) or two preparations (●, ■).

Fig. 2. Effect of CCCP on the uptake of amino acids and sucrose by vesicles of the plasma membrane-enriched fraction. Uptake was determined after 4 min of incubation in pH5Na-medium without (black columns) or with 10 μM CCCP (light grey columns), or in pH7K-medium without (dark grey columns) or with 10 μM CCCP (white columns). Bars represent mean values ± SE of two (l-valine, l-lysine) or three (l-glutamate, sucrose) preparations. Uptakes of lysine in pH5Na with CCCP and in pH7K were determined in a single preparation. Substrates were supplied to the uptake media in the following concentrations: l-valine, 1.12 μM; l-lysine, 0.85 μM; l-glutamic acid, 1.04 μM; sucrose, 0.76 μM.

Uptake of sucrose

As indicated by the intercepts of the uptake-time curves on the y-axis, an appreciable part of the sucrose uptake (about 9 pmol mg⁻¹ protein) appeared to be time-independent (Fig. 4). In these experiments this amount was equivalent to ~80 dpm or ~0.06% of the radioactivity in the 0.2 ml samples of the incubation mixture from which the vesicles were collected in the uptake assay. Most likely, it represents residual extravasculat label.

Imposition of $\Delta \Delta \psi$ stimulated the uptake of sucrose, whereas the imposition of $\Delta \psi$ had no detectable effect.

Fig. 3. Accumulation ratios for the uptake of amino acids by vesicles of the plasma membrane-enriched fraction. Uptake was measured after 4 min of incubation in the pH5Na-medium (imposition of $\Delta \Delta \psi$) and concentrations of the amino acids in the vesicles were calculated assuming an intravesicular volume of 5 μl mg⁻¹ protein. Bars represent mean values ± SE of two determinations with a single preparation (l-ala, l-thr, l-arg) or correspond to the uptakes as shown in Fig. 1 (l-val, l-lys, l-glu). The amino acids were supplied to the uptake medium in the following concentrations (μM): l-alanine, 25; l-threonine, 35; l-valine, 1.12; l-lysine, 0.85; l-arginine, 0.60; l-glutamic acid, 1.04.

The $\Delta \Delta \psi$-dependent sucrose uptake, which could be completely abolished by CCCP (Fig. 2b), reached a level of ~12 pmol mg⁻¹ protein, corresponding to an accumulation ratio of about 3.

Discussion

$H^+$-symport of amino acids and sucrose

Transport of the amino acids l-valine, l-lysine and l-glutamic acid into vesicles from the plasma membrane-enriched fraction is by a $H^+$-symport mechanism as evidenced by the stimulating effect of $\Delta \Delta \psi$. Furthermore, the transport of valine and lysine appears to be electrogenic since it could be enhanced by the imposition of $\Delta \psi$. The influx of valine was stimulated 2–3-fold by $\Delta \psi$, which is similar to that of the low-affinity $H^+/amino$
acid symporters AAP1 and AAP5 from Arabidopsis (Boorer et al., 1996; Boorer and Fischer, 1997).

Transport of lysine was more strongly stimulated by Δψ. Thus the ΔpH-dependent uptake of lysine increased about 6 times when Δψ was imposed simultaneously, as has been found in plasma membrane vesicles from developing cotyledons (De Jong and Borstlap, 2000). But in contrast with the vesicles from cotyledons, those isolated from the seed cores clearly showed a ΔpH-stimulated uptake of L-glutamic acid (Fig. 1c). The imposition of Δψ had no effect which suggests an electroneutral transport of anionic glutamate with one proton. Up to now the transport of glutamic acid across the plant plasma membrane has been found to be electrogenic, probably as a result of the symport of anionic glutamate with two protons (Kinraide and Ethanor, 1980; Wyse and Komor, 1984; Weston et al., 1995). On the other hand, it has been suggested that the neutral form of glutamic acid is the substrate for broad specificity amino acid transporters (Borstlap et al., 1986). Compelling evidence has been obtained that AAP5, a broad specificity amino acid transporter from Arabidopsis, transports the neutral form of glutamic acid with one proton (Boorer and Fischer, 1997).

As in vesicles from cotyledons, the uptake of sucrose in membrane vesicles from seed coats was much lower than that of amino acids and an effect of Δψ could not be detected. These findings have been discussed elsewhere (De Jong and Borstlap, 2000). The presence of the activity of an H⁺-sucrose symporter in the seed coat is in line with the results of molecular studies. VISUT1 from broad bean and PsSUT1 from pea, the sucrose transporters in developing cotyledons, have also been found to be expressed in developing cotyledons (Weber et al., 1997; Tegeder et al., 1999).

**Comparison with the uptake of sucrose and amino acids by isolated seed coat halves**

During the later stages of the developing pea seed the endosperm has disappeared and the assimilates released by the seed coat can be absorbed directly by the cotyledons. Proton symporters are involved in transport of sucrose and amino acids into the cotyledons (De Jong and Borstlap, 2000). But it is unlikely that symporters are also employed for the release of their substrates from the seed coat parenchyma cells, since the release was not enhanced by CCCP (De Jong and Wolsinkwinkel, 1995). Furthermore, the uptake of sucrose, L-valine and L-glutamic acid by isolated seed coat halves of developing pea seeds showed strict linear kinetics and was insensitive to CCCP. Uptake of L-lysine displayed an apparent saturable component which was neither inhibited by CCCP and has been proposed to result from the interaction between Δψ and the unipor of the cationic amino acid. From these results it has been concluded that seed coat parenchyma cells are not equipped with H⁺-symporters for sucrose and amino acids, which would nicely fit with their function as solute-releasing cells (De Jong et al., 1996, 1997). Surprisingly, the results of the present study clearly demonstrate the presence of H⁺-symporters for sucrose and amino acids in the pea seed coat. How can these seemingly contradictory results be reconciled?

First, it is possible that the plasma membrane-localized H⁺-symporters are restricted to cell types of the seed coat that cannot be reached by the exogenously supplied substrates. This seems unlikely, however, because there is no evidence for apoplastic barriers in the seed coat.

Secondly, it could be that H⁺-symporters for sucrose and amino acids are present in the plasma membranes of seed coat cells, but that for some reason their activity was not detected in the uptake experiments with the isolated seed coat halves. Again this is not very probable because the activity of H⁺-symporters for sucrose and amino acids can be easily demonstrated in isolated cotyledons, even though the activity of the symporters in plasma membrane vesicles from cotyledons was two to four times lower than in vesicles from the seed coats (De Jong and Borstlap, 2000).

Thirdly, the symporters may be localized in one of the endomembranes. Indeed, the relatively high enrichment of the bafilomycin-inhibitable ATPase activity in the plasma membrane fraction from pea seed coats points to a considerable contamination with a membrane that contains a V-type ATPase, most likely the tonoplast. However, no H⁺-symporters for sucrose and amino acids...
are known that localize to the tonoplast or to any other endomembrane of the plant cell. Sucrose is probably transported across the tonoplast by an H⁺/sucrose antiporter (Getz and Klein, 1995) whereas amino acid transporters of the tonoplast seem to be of a completely different type (Martinoia et al., 1992; Dietz et al., 1994).

Finally, it could be envisaged that genes encoding the H⁺-symporters are expressed in the seed coat tissue, but that the proteins are not targeted properly to the plasma membrane. Thus the symporters may be present, in a fully active form, in vesicles of the secretory pathway. If true, this suggests that the functional expression of transporters destined for the plasma membrane may be regulated at the level of protein trafficking.

**Concluding remarks**

The presence of H⁺-symporters for sucrose and amino acids has been demonstrated in a plasma membrane-enriched fraction from developing pea seed coats. Though no definite conclusion can be drawn, this result points to either of two possibilities. (1) The H⁺-symporters are localized in the plasma membrane of seed coat cells, but for some reason their activity has not been detected in uptake experiments with seed coat halves (De Jong et al., 1996, 1997). In as much as the seed coat releases sucrose and amino acids this would imply some degree of futile cycling of these substrates across the plasma membrane of seed coat parenchyma cells. (2) The H⁺-symporters reside in one of the intracellular membranes. It is tempting to speculate that the symporters remain in vesicles of the secretory pathway that fail to target properly to the plasma membrane.

**Note added in proof**

Recently, the isolation of PsAAP1 from a cotyledon cDNA library of pea was reported. PsAAP1 belongs to the AAP family of H⁺/amino acid symporters and mediates transport of neutral, acidic, and basic amino acids. Transcripts of PsAAP1 were also detected in coats of developing seeds (Tegeder et al., 2000).

**References**


