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# Amino Acid Transport into Membrane Vesicles Isolated from Zucchini[1]

## EVIDENCE OF A PROTON-AMINO ACID SYMPORT IN THE PLASMALEMMA

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### ABSTRACT

**Several lines of evidence with intact tissues suggest amino acid transport is mediated by a proton-amino acid symport (L Rheinhold, A Kaplan 1984 Annu Rev Plant Physiol 35: 45–83). However, biochemical studies of proton-coupled amino acid transport in isolated membrane vesicles have not been reported. In the experiments presented here, amino acid transport was studied in membrane vesicles isolated from zucchini (*Cucurbita pepo* L. cv Black Beauty) hypocotyls. An imposed pH gradient (basic interior) was used to energize isolated membrane vesicles and drive amino acid transport. Proton-coupled amino acid accumulation was demonstrated for alanine, glutamate, glutamine, leucine, and tabtoxinine-β-lactam. Alanine transport into the isolated membrane vesicles was studied in detail. Alanine transport was protonophore sensitive and accumulation ratios exceeding 10 times that predicted by diffusion alone were observed. ΔpH-Dependent alanine transport exhibited saturation kinetics, suggesting translocation was mediated via a carrier transport system. In support of that conclusion, 50 micromolar *N,N'*-dicyclohexylcarbodiimide, a hydrophobic modifier of protein carboxyls, completely inhibited proton-coupled alanine accumulation. Transport activity, equilibrated on a linear sucrose gradient, peaked at 1.16 grams per cubic centimeter and co-migrated with a plasmalemma marker (vanadate-sensitive $K^+$-$Mg^{2+}$-ATPase). These results provide direct evidence in support of a proton-amino acid symport in the plasmalemma of higher plants.**

Amino acid transport is a fundamental process required for normal growth and development in the heterotrophic tissues of the plant, including the root, expanding leaves, and developing fruit. Amino acid uptake is considered a secondary active transport process coupling amino acid influx to the pmf[3] (17) across the plasmalemma (7, 10, 11, 20, 24). Thus, amino acid translocation into the cell is linked to the energetically 'down hill' flux of protons into the cytoplasm.

The plasmalemma and tonoplast membranes in higher plants are responsible for transporting both organic and inorganic nutrients into and out of the cytoplasm. The free energy required for this transport is derived from proton electrochemical gradients generated by two distinct electrogenic proton-pumping ATPases: one on the plasmalemma and the other on the tonoplast (24). In addition, the tonoplast contains a proton-pumping pyrophosphatase that may contribute to the pmf across this membrane (19). Several proton-coupled transport systems of the tonoplast have been described using isolated membrane vesicles (1, 24). In contrast, biochemical studies of proton-coupled nutrient transport in isolated plasmalemma vesicles have not been reported. Nevertheless, inhibitor effects and electrophysiological experiments with intact cells correlate nutrient transport across the plasmalemma with the pmf (20, 24).

We are interested in amino acid transport as a fundamental transport activity and because the chlorosis-inducing toxin tabtoxinine-β-lactam, a novel amino acid and glutamine synthetase inhibitor (12) produced by the plant pathogen *Pseudomonas syringae* pv tabaci, is transported into the plant cell by an amino acid transport system (3, 4). Here we report on experiments using isolated membrane vesicles and imposed proton gradients to examine proton-coupled amino acid transport at the plasmalemma. Our results provide direct evidence in support of the chemiosmotic model of amino acid transport into higher plant cells.

## MATERIALS AND METHODS

**Materials.** Seedlings of zucchini (*Cucurbita pepo* L. cv Black Beauty) were grown in the dark for 7 d in moist vermiculite at 25°C. [$^{14}$C]Amino acids were obtained from New England Nuclear. Sodium-ATP was converted to Btp-ATP with Dowex ion exchange resin and subsequent titration with solid Btp to the desired pH. All chemicals were reagent grade.

**Isolation of Membrane Vesicles.** Membrane vesicles were isolated from etiolated hypocotyl tissues using a protocol based on the method of Clark and Goldsmith (6). All isolation steps were conducted at 4°C. Hypocotyl tissue was homogenized with a mortar and pestle in 250 mM sorbitol, 50 mM Hepes, 3 mM EGTA, 2 mM DTT, 1 mM HCl, and 0.5% BSA (10 mL/g fresh weight). Homogenization solution pH was adjusted to 8.0 with solid Btp. Mitochondria were removed from filtered homogenate (4 layers cheesecloth) by centrifugation for 10 min at 12,000*g*. Microsomal membranes were isolated from the supernatant by a subsequent 35 min centrifugation at 60,000*g*. The membrane vesicles were then washed and repelleted twice: first in 36 mL of the homogenization solution and then in 36 mL of resuspension buffer (350 mM sorbitol, 2.5 mM Hepes, 1 mM HCl, 0.1 mM DTT, and pH adjusted to 8.0 with Btp). The final pellet was resuspended in 1 mL of resuspension buffer per 5 g starting

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[3] Abbreviations: pmf, proton motive force; Btp, 1,3-bis-(tris[hydroxymethyl]methylamino)propane; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

material (about 1 mg/mL). Chloride, a mobile anion in plant membrane vesicles, was included in all solutions to avoid development of significant membrane potentials.

When transport activity was measured across linear sucrose gradients, the above isolation protocol was used except sucrose was substituted for sorbitol in both the homogenization and resuspension solutions. The resuspended microsomal membranes were layered over a linear sucrose gradient (20–40%, in 2 mM Hepes, 1 mM HCl, 1 mM DTT, and pH adjusted with Btp to 8.0), centrifuged for 3 h at 100,000*g*, and then collected in 1 mL fractions.

**Amino Acid Transport.** Transport experiments were conducted at 10 to 14°C. Transport was initiated by diluting a small aliquot (20–50 μL) of resuspended membrane vesicles into an acidic transport solution (600 μL) containing resuspension buffer, (pH adjusted to 6.0 with solid Mes, about 10 mM), 0.1 to 0.5 μCi [$^{14}$C]amino acid, and unlabeled amino acid to the desired final concentration. At predetermined time points, membranes were collected and washed using a Millipore filtration technique (5). A slightly different protocol was used when examining the kinetics of alanine transport. The membranes were added to the acidic transport solution in the absence of alanine. After 2 min, 0.1 to 0.5 μCi [$^{14}$C]alanine and unlabeled alanine were added to the desired final concentration. This procedure was necessary to ensure linear rates of transport. Lomax and Melhorn (13), working with zucchini membrane vesicles, showed that the transmembrane proton gradient partially collapses during the first minute after jumping. The gradient then remains constant for as long as 1 h. Preliminary data using $^{14}$C-acetate distribution to probe the pH gradient established in our membrane vesicles confirmed this observation (data not shown). Therefore, it was necessary to generate a stable proton gradient before examining transport kinetics. With this prejump protocol, alanine transport was linear for at least the first two sample times at all concentrations tested. Accumulated radioactivity was measured with scintillation spectrometry. Each experiment was repeated at least twice. Standard errors never exceeded ±5% of experimental averages. The results presented are from one representative experiment.

**Enzyme Assays and Protein Determination.** ATPase assays were conducted following the method of Buckhout and Hrubec (2). Specific assays included: (a) Vanadate-sensitive, $K^+$-$Mg^{2+}$-ATPase; 30 mM Hepes-Btp (pH 6.8), 25 mM $K_2SO_4$, 3 mM $MgSO_4$, 3 mM Btp-ATP (7.0), 0.02% (v:v) Triton X-100, and ± 100 μM $Na_3VO_4$; (b) nitrate-sensitive, $Cl^-$-$Mg^{2+}$-ATPase; 30 mM Hepes-Btp (pH 8.0), 50 mM KCl, 3 mM $MgSO_4$, 3 mM Btp-ATP (7.8), and ±50 mM Btp-$NO_3$. Antimycin A-insensitive, NADH-dependent Cyt *c* reductase was assayed according to Lord *et al.* (14). Proteins were determined with the Lowry method (15) after solubilization in 0.15% Na deoxycholate and precipitation in cold 12% TCA.

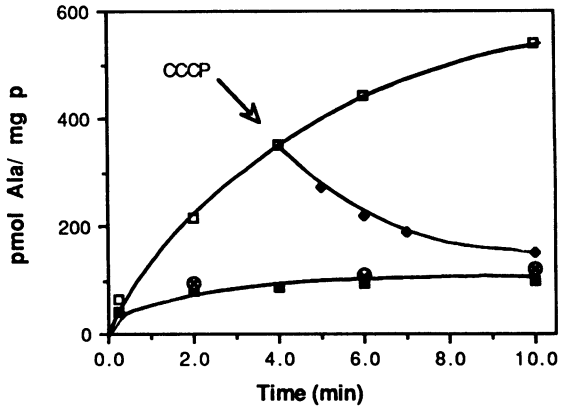


FIG. 1. Proton gradient-dependent [$^{14}$C]alanine transport into zucchini membrane vesicles. A small aliquot (20–50 μl) of resuspended membranes (pH 8.0) were diluted into an acidic transport solution (600 μl) containing resuspension buffer (pH adjusted to 6.0 with solid Mes), 3 μM [$^{14}$C]alanine, and plus (■) or minus (□) 5 μM CCCP. For one treatment, CCCP was added 4 min after adding the membranes (◆). Transport in the absence of a proton gradient (pH 8.0) into 8.0 (○) or 6.0 into 6.0 (×), is also shown. Membranes equilibrated at pH 6.0 were produced with a single freeze-thaw cycle. ΔpH-Dependent alanine transport was unaffected in pH 8.0 membranes exposed to the same treatment (12 h at −20, 10 min at 25°C) before the pH jump.

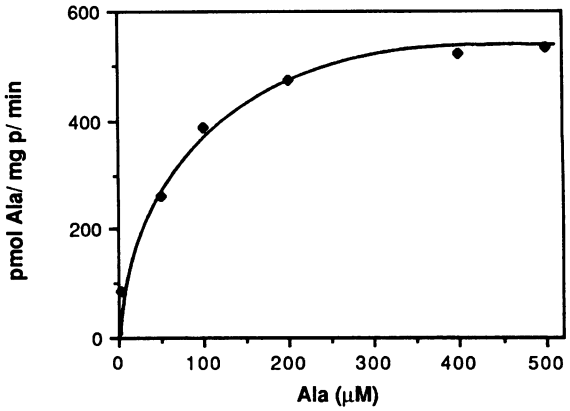


FIG. 2. Effects of alanine concentration on the ΔpH-dependent uptake of [$^{14}$C]alanine into isolated membrane vesicles. The membranes and transport solutions were as in Figure 1. For these experiments, the membrane vesicles were added to the acidic resuspension buffer for 2 min before adding alanine. Under these conditions, a constant pmf was established and linear rates of transport were recorded (see "Materials and Methods").

## RESULTS AND DISCUSSION

To study proton-coupled amino acid transport, we used imposed pH gradients to energize isolated membrane vesicles. Zucchini membrane vesicles (6, 8) were used because they maintain pH gradients for as long as 1 h. Vesicles were isolated and purified in solutions buffered at pH 8.0. A proton electrochemical gradient was formed across the vesicles' membrane by diluting them into an acidic (pH 6.0) transport solution. This procedure produces a transmembrane proton gradient (basic interior) that is in the same orientation as that found across the plasmalemma *in vivo*. Under these conditions, we reasoned, plasmalemma vesicles in the native configuration (outside-out) should be competent in proton-coupled amino acid transport. ΔpH-dependent transport of alanine is shown in Figure 1. Alanine accumulation was dependent on the pH gradient as illustrated by the absence of transport activity in the presence of CCCP, a proton ionophore. The release of accumulated alanine when CCCP was added after 4 min of active transport, also demonstrates that alanine accumulation is dependent on the proton gradient. In the absence of an imposed proton gradient, alanine accumulated to the same level as that measured in the presence of CCCP (Fig. 1). The estimated intravesicular concentrations of alanine, assuming a vesicle volume of 10 μL/mg protein (13), were more than an order of magnitude higher than that predicted by simple diffusion. Thus, ΔpH-dependent alanine accumulation is against a significant concentration gradient.

We investigated the kinetics of ΔpH-dependent alanine transport to differentiate between diffusion through the lipid bilayer and protein-mediated transport. The observed saturation kinetics (Fig. 2) are consistent with a carrier-mediated pathway that saturates because it has a finite number of entry sites. In further

support of carrier-mediated transport, the ΔpH-dependent alanine accumulation was completely inhibited by a 30 min pretreatment in 50 μM *N,N'*-dicyclohexylcarbodiimide, a hydrophobic modifier of protein carboxyls. This is of particular significance because N,N'-dicyclohexylcarbodiimide is a potent inhibitor of several other proteins involved with proton translocation, including the $F_0F_1$ATPase (23) and the proton-calcium antiport of the plant tonoplast (5, 22). These results, taken together with the proton gradient-dependence, provide good evidence for proton symport-mediated amino acid transport in zucchini membrane vesicles.

The proton-amino acid symport was associated with plasmalemma vesicles based on several experimental criteria. The protonophore-sensitive alanine transport activity, equilibrated on a linear sucrose gradient, peaked at a density of 1.16 g/cc (Fig. 3*A*). This density agrees with published estimates of plasmalemma density on sucrose gradients (9, 18, 24). Furthermore, this transport activity co-migrated with a plasmalemma marker (vanadate-sensitive $K^+$-$Mg^{2+}$-ATPase activity), while it was separated from the tonoplast and endoplasmic reticulum membranes, based on marker distribution and membrane density (5, 16, 18) (Fig. 3, A and B). A plasmalemma location for this transport system is also consistent with electrophysiological studies correlating plasmalemma membrane potential changes with amino acid transport (10, 20).

ΔpH-Dependent amino acid transport, as measured by protonophore sensitivity, was also demonstrated in this study for glutamate, glutamine, leucine, and tabtoxinine-β-lactam (Table I). These amino acids represent a range of charge and structural configurations. Whether or not they are transported by a single transport protein or several different proteins has not been determined. Nevertheless, protonophore sensitivity couples their translocation into the cell to the proton motive force.

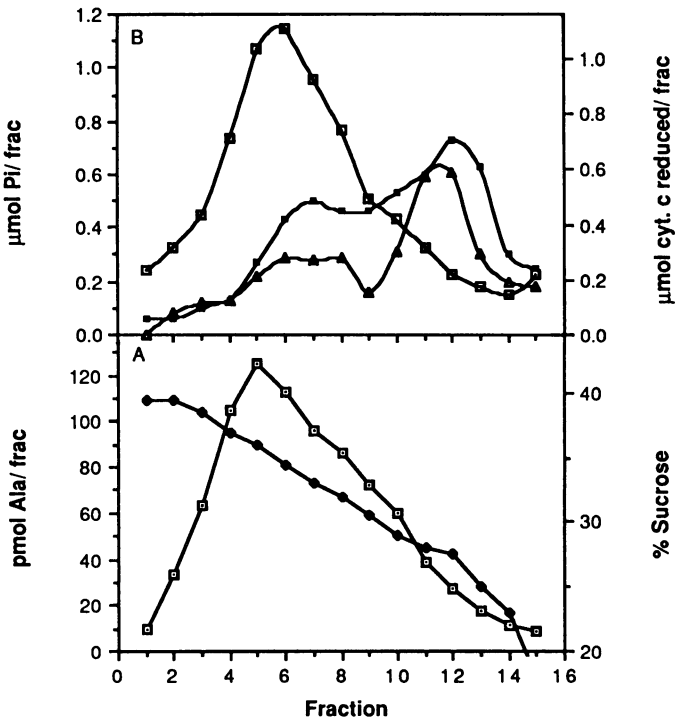


FIG. 3. Distribution of ΔpH-dependent [$^{14}$C]alanine uptake and enzyme activities associated with the plasmalemma, tonoplast, and endoplasmic reticulum. A, (⊡), protonophore-sensitive alanine uptake; (◆), sucrose concentration. B, Membrane marker distributions: (□), plasmalemma (18, 24), vanadate-sensitive $K^+$-$Mg^{2+}$-ATPase; (△), tonoplast (5, 16, 24), nitrate-sensitive $Cl^-$-$Mg^{2+}$-ATPase; (■), endoplasmic reticulum (11), antimycin A-insensitive NADH-dependent Cyt *c* reductase.

Table I. *Protonophore-Sensitive Amino Acid Transport into Isolated Microsomal Membrane Vesicles*

Transport experiments with $^{14}$C-labeled amino acids were conducted as described in "Materials and Methods." The transport period was 4 min for glutamate, glutamine, and leucine and 30 min for tabtoxinine-β—lactamPlus protonophore included 5 μM CCCP. [$^{14}$C]Tabtoxinine-β-lactam was prepared as described in Bush and Langston-Unkefer (3).

| Amino Acid | Control | Plus CCCP | Sensitive |
|---|---|---|---|
| | *pmol/mg protein/min* | | % |
| Glutamate, 5 μM | 9.68 | 4.02 | 58 |
| Glutamine, 5 μM | 44.1 | 17.2 | 61 |
| Leucine, 5 μM | 51.3 | 19.0 | 63 |
| Tabtoxinine-β-lactam, 5 mM | 189 | 101 | 46 |

Alanine transport was also examined in membrane preparations in which NaOH was used in place of Btp to titrate the homogenization and resuspension buffers. Membrane vesicles isolated in sodium based solutions exhibited a 36% decrease in proton coupled alanine transport activity when compared to membranes isolated in the presence of Btp. Several explanations could account for this decrease, including a sodium effect on the transport protein, an indirect effect on the pmf, or an indirect effect of ionic strength exerted during isolation. Schneegurt and McDaniel (21) have reported significant decreases in leucine transport into cultured tobacco cells when Btp or other amine based buffers were used in their transport solutions. In the presence of nonamine buffers and NaOH, transport was not diminished. The results presented here suggests the effect of Btp on leucine transport in intact cells may be the result of an indirect action on the plasmalemma and not on leucine transport *per se*. Resolution of these observations requires a more thorough examination of amino acid transport in both isolated membrane vesicles and intact cells.

## CONCLUSION

Amino acid transport into the plant cell is generally thought to be mediated by a proton-amino acid symport (20). This conclusion is based upon studies with intact tissues and cells that exhibit transport characteristics that are consistent with this hypothesis. However, some of the results cited as evidence in support of this conclusion have been questioned (20). Perhaps the most convincing evidence derived from intact tissue is found in electrophysiological experiments in which amino acid transport into the cell is correlated with depolarization of the membrane potential (7, 10, 11). Transient membrane depolarization is expected if amino acid import is coupled to the simultaneous influx of protons. The influx of positive charge and the resulting decrease in membrane potential appears to be compensated for by higher rates of proton-pumping by the plasmalemma ATPase (10).

In the results presented here, we have provided direct evidence in support of a proton-amino acid symport in the plant plasmalemma using isolated membrane vesicles and imposed proton electrochemical gradients. This experimental approach has unequivocally coupled amino acid transport with proton flux. Specific knowledge about the number of transport proteins, the mechanism coupling translocation to proton flux, and the biochemical regulation of amino acid transport is not available. In future work, the vesicle approach will be used to identify the transport protein(s) and to address specific questions regarding the structure, function, and regulation of membrane proteins mediating amino acid transport into the cell.

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