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Uptake of Phenylalanine into Isolated Barley Vacuoles Is Driven by Both Tonoplast Adenosine Triphosphatase and Pyrophosphatase[1]

Evidence for a Hydrophobic L-Amino Acid Carrier System

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ABSTRACT

The uptake of phenylalanine was studied with vacuole isolated from barley mesophyll protoplasts. The phenylalanine transport exhibited saturation kinetics with apparent K_m-values of 1.2 to 1.4 millimolar for ATP- or PPi-driven uptake; $V_{max\ app}$ was 120 to 140 nanomoles Phe per milligram of chlorophyll per hour (1 milligram of chlorophyll corresponds to 5×10^6 vacuoles). Half-maximal transport rates driven with ATP or PPi were reached at 0.5 millimolar ATP or 0.25 millimolar PPi. ATP-driven transport showed a distinct pH optimum at 7.3 while PPi-driven transport reached maximum rates at pH 7.8. Direct measurement of the H^+-translocating enzyme activities revealed $K_{m\ app}$ values of 0.45 millimolar for ATPase (EC 3.6.1.3) and 23 micromolar for pyrophosphatase (PPase) (EC 3.6.1.1). In contrast to the coupled amino acid transport, ATPase and PPase activities had relative broad pH optima between 7 to 8 for ATPase and 8 to 9 for PPase. ATPase as well as ATP-driven transport was markedly inhibited by nitrate while PPase and PPi-coupled transport was not affected. The addition of ionophores inhibited phenylalanine transport suggesting the destruction of the electrochemical proton potential difference $\Delta \mu H^+$ while the rate of ATP and PPi hydrolysis was stimulated. The uptake of other lipophilic amino acids like L-Trp, L-Leu, and L-Tyr was also stimulated by ATP. They seem to compete for the same carrier system. L-Ala, L-Val, D-Phe, and D-Leu did not influence phenylalanine transport suggesting a stereospecificity of the carrier system for L-amino acids having a relatively high hydrophobicity.

Vacuoles are the largest organelles of differentiated plant cells occupying up to 90% of the total cell volume. As reviewed by Boller and Wiemken (4), they fulfill a series of functions. Active transport into this compartment has been demonstrated for metabolic intermediates as well as inorganic ions. Phenylalanine, the major aromatic amino acid synthesized in chloroplasts (3), is transiently stored in the vacuole. Up to 95% of all free cellular phenylalanine is found in this compartment (2; and E Martinoia, personal communication). Transport is an active, ATP consuming process which is coupled to an electrochemical proton potential difference $\Delta \mu H^+$ (10). In *Saccharomyces cerevisiae* the existence of at least seven independent proton/amino acid antiport systems could be demonstrated (21), each fueled by ATP. As reviewed by Rea and Sanders (20), energization of the tonoplast membrane could be fulfilled by two distinct enzyme activities; the tonoplast H^+-translocating ATPase and PPase. In this report we present data that uptake of phenylalanine into vacuoles, isolated from barley mesophyll protoplasts, can be driven by both H^+-translocating enzymes as distinguished by their differential sensitivity to nitrate and different pH optima. Moreover, the involved carrier system seems to be a type having a high affinity for the lipophilic L-amino acids.

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MATERIALS AND METHODS

Chemicals

If not stated otherwise all chemicals were from Sigma Chemie, Deisenhofen, FRG; Boehringer, Mannheim, FRG; or Merck, Darmstadt, FRG and are of highest analytical grade.

Radiochemicals

L-[U-^{14}C]phenylalanine (19.0 GBq·mmol^{-1}), L-[U-^{14}C]leucine (12.7 GBq·mmol^{-1}), L-[U-^{14}C]tyrosine (19.0 GBq·mmol^{-1}), L-[methylene-^{14}C]tryptophane (2.18 GBq·mmol^{-1}), ^3H_2O and ^{32}P were from Amersham Buchler, Braunschweig, FRG.

Plant Material

Hordeum vulgare cv Lilo was from Saaten Union Hannover, FRG; cv Gerbel was from von Lochow-Pettkus, Bergen, FRG. Plants were grown as previously described (10).

Preparation of Vacuoles

Vacuoles from barley mesophyll protoplasts were prepared according to Kaiser *et al.* (13) with modification (10). The number of the floated vacuoles was estimated with the assumption that 10^7 vacuoles have a total volume of 164 μL

(10). For conversion, $5 \cdot 10^6$ protoplasts correspond to 1 mg Chl (10).

Chl Determination

Chl was determined according to Arnon (1).

Assays

Synthesis of γ-^{32}P-labeled ATP was done as previously described (8); for measurement of the ATPase activity the method of Franek and Strotmann (8) was modified as follows: The samples were mixed with 5 mL of a solution containing 1% ammonium molybdate in 1 M $HClO_4$. Of the mixture, 100 μL were taken to determine the total activity of ^{32}P. The remaining volume was mixed with 2 mL of 2-methyl-1-propanol:toluene (1:1, v/v); 0.5 mL of the organic phase was used for determination of the hydrolysed ^{32}P. Prior to the experiments, in order to avoid volume errors, the organic medium was saturated with water and the molybdate medium was saturated with the organic mixture.

ATPase and PPase[2] were measured as follows: Purified vacuoles were incubated in a medium containing 25 mM Hepes/KOH (pH 7.4), 10 mM $MgCl_2$, 50 mM KCl, 0.6 M sorbitol, and 0.5 mM [γ-^{32}P]ATP or 2 mM sodium pyrophosphate. Aliquots were taken after 5, 10, 20, and 30 min and the reaction was terminated by addition of $HClO_4$ (final concentration 0.3 M). The release of ^{32}P was determined by liquid scintillation counting in a Packard scintillation counter 3255. Hydrolysis of PPi was measured by release of Pi (7).

For transport studies, 70 μL incubation medium containing 40% Percoll, 0.45 mM sorbitol, 30 mM Hepes/KOH (pH 7.4 [ATP-driven transport] or 7.8 [PPi-driven transport]), 0.5% purified BSA, 5 mM DTT, 3.1 KBq ^{14}C-labeled amino acids, 5.3 KBq ^3H_2O and solutes as indicated in the figures and tables were pipetted into a 400 μL Beckmann tube. Thirty μL of a vacuolar suspension were added and overlayered with 200 μL silicon oil AR 200 (Wacker Chemie, München, FRG) and 60 μl H_2O. All experiments were performed as kinetics with time steps at 5.5, 10, 14.5, 19, 23.5 min, and transport rates were calculated by linear regression. The transport was linear for at least 30 min. At the indicated times the tubes were centrifuged for 15 s at 14,000g in a Beckmann Microfuge E and 50 μL aliquots of the supernatant were counted in a Beckmann scintillation counter LS 1801. The number and the volume of the floated vacuoles were estimated in principal as described by Strotmann and Thiel (24).

The relative rate of the hydrophobicity of the amino acids was attributed to their interaction with C_{18}-groups on a reversed phase HPLC column or on reversed phase thin layer plates. The HPLC system consisted of a Beckmann 110 B solvent delivery module, a Beckmann 163 variable wavelength detector, and a Beckmann Ultrospec ODS RP 18, 5 μm, 4.6·250 mm column. Ten mM KH_2PO_4 (pH 4.0), was used as solvent (flow rate 2 mL·min^{-1}). The aromatic amino acids were detected at 214 nm, the others at 195 nm. The relative hydrophobicity $x = 1$ based on the retention time of Phe (R_T = 10.4 min). The following values (x) were obtained: Ala, 0.105; Val, 0.152; Ile, 0.286; Leu, 0.305; Tyr, 0.390; Trp, 2.790.

TLC was performed on Merck HPTLC RP-18 $W_{254}S$ plates with 10 mM KH_2PO_4 (pH 7.4) as solvent. The relative hydrophobicity $x^* = 1$ based on the R_f^{-1}-value of Phe ($R_f^{-1} = 2.78$). The following values of x^* were obtained: Ala, 0.41; Val, 0.50; Ile, 0.60; Leu, 0.64; Tyr, 0.77; Trp, 2.12. The increasing hydrophobicity from Ala to Trp is in good agreement with the hydrophobicity scale presented by Nozaki and Tanford (18).

RESULTS

Table I shows the influence of several nucleotide species on rates of phenylalanine uptake. Similar to results for malate transport presented by Martinoia *et al.* (16), the uptake of phenylalanine was also driven by nucleotides other than ATP. This agrees with direct measurement of the ATPase activity (19) as well as the membrane bound enzyme (26) and reveals that the enzyme has minor affinities for related nucleotides. Beside nucleotide-stimulated transport, phenylalanine uptake is markedly accelerated by PPi, indicating the participation of the tonoplast-bound PPase. Addition of 40 mM nitrate (Table II) markedly inhibited ATPase-driven transport while PPase-driven transport was not affected. Vanadate as well as azide had no effect or only a slight influence on rates of transport. Ionophores such as nigericin, gramicidin, and valinomycin greatly inhibited ATP- and PPi-driven phenylalanine transport, indicating the participation of both Δ pH and $\Delta \mu H^+$ in this process. Probably the primary target is the Δ pH, which can be dissipated by valinomycin via the tonoplast H^+/K^+ antiporter.

Direct measurement of the enzymes revealed that the ATPase is inhibited by nitrate while vanadate and azide showed no effect. No influence of these three substances on PPase activity could be detected. Destroying the transmembrane

Table I. *Influence of Several Nucleotide Species on Rates of Phenylalanine Transport*

Rates were measured at pH 7.4 for ATPase and at pH 7.8 for PPase. Nucleotides (5 mM) were added as Mg-salts (for ATP) or equal amounts of Mg-gluconate were additionally added to the assay medium containing 1 mM L-phenylalanine. For PPi, a 10-fold surplus of Mg-gluconate and 40 mM K-gluconate were added to the assay medium. In all cases a basal rate in the range of 40% of the maximal rate was subtracted. This basal rate represents the rate of transport without addition of any nucleotide species. Relative rate 1 was (in nmol L-Phe·mg Chl^{-1}·h^{-1}) 56.45 or, in the case of MgPPi*, 63.76. Transport was linear for at least 30 min (data not shown). Rates were calculated by linear regression and the assumption that 1 mg Chl corresponds to $5 \cdot 10^6$ vaculoes.

Nucleotide Species	Relative Rate
MgATP	1
MgXTP	0.27
MgGTP	0.37
MgUTP	0.34
MgCTP	0.14
MgPPi	0.67

[2] Abbreviations: PPase, pyrophosphatase; *p*-F-Phe, *p*-fluorophenylalanine; Phe-Pry, phenylpyruvate; hyd, hydrolyzed; XTP, xanthosine 5′-triphosphate; app, apparent.

Table II. *Influence of Inhibitors on L-Phenylalanine Transport (A) and Enzyme Activity of Tonoplast ATPase and PPase (B)*

For transport experiments (A) the assay medium contained 1.5 mM L-Phe (1 mM in *), 10 mM MgATP or 0.5 mM PPi plus 5 mM Mg-gluconate, and 40 m*M* K-gluconate. pH values were 7.4 for ATPase and 7.8 for PPase. 100% expresses (in nmol L-Phe·mg $Chl^{-1}\cdot h^{-1}$) 62.8 (46.2 in *) for ATPase and 90.7 for PPase. Because the effect of nigericin is considerably decreased by the addition of BSA (data not shown) only traces of BSA had been given to the assay medium. Gluconate (50 mM K+) were additionally added to assay medium containing nigericin, valinomycin and gramicidin. The enzyme activities (B) were assayed as described in "Materials and Methods." The incubation was performed at the same pH values as in A. Rate (100%) was (in μmol ATP or $PPi_{hydrolysed}$·mg $Chl^{-1}\cdot h^{-1}$) 12.0 for ATPase and 3.75 for PPase. The transport rates were calculated as described in Table I.

Substrate	A Transport		B Enzyme activity	
	ATPase	PPiase	ATPase	PPiase
	%		%	
+ ATP (10 mM)	100*	−/−	100	−/−
− ATP	41.1*	−/−	ND[a]	−/−
+ PPi (0.5 mM)	−/−	100	−/−	100
− PPi	−/−	46.5	−/−	ND
+ Nitrate (40 mM)	49.2*	98.2	52	89
+ Azide (1 mM)	87*	ND	92	84
+ Vanadate (0.4 mM)	110*	ND	97	84
+ Oligomycin (40 μg/mL)	101	ND	ND	ND
+ Nigericin (10 μM)	49.5	50.2	194	198
+ Valinomycin (5 μM)	39.9	48.3	227	185
+ Gramicidin (5 μM)	55.6	43.6	212	221

[a] Not determined.

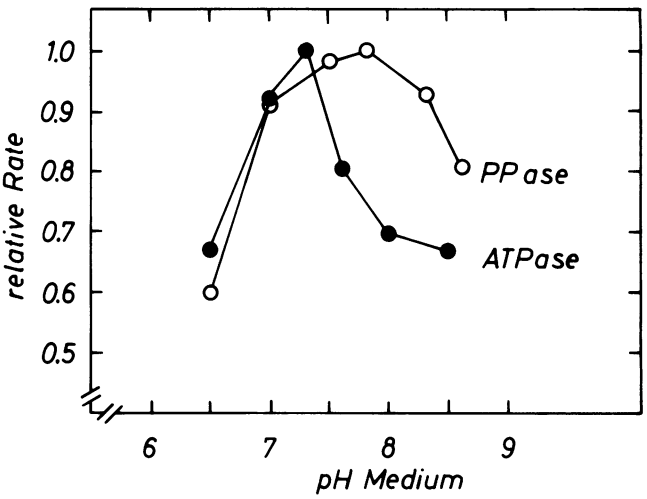

Figure 1. Influence of external pH on rates of ATPase- and PPase-driven phenylalanine uptake into isolated vacuoles. The assay medium contained 80 mM buffer at the indicated pH-value (Mes for pH value below 7.0; Hepes for pH value above 7.0), 1.5 mM L-phenylalanine and 5 mM MgATP or 0.5 mM PPi plus 5 mM Mg^{2+} (added as gluconate). The buffer capacity of the vacuolar suspension, added to the assay medium, was lowered to 5 mM Hepes/KOH (pH 7.4 or 7.8). The addition of vacuoles did not cause significant pH changes. Relative rate 1 expresses (in nmol L-Phe·mg $Chl^{-1}\cdot h^{-1}$) 64.2 for ATPase at pH 7.3; 96.7 for PPase at pH 7.8. The rates were calculated as described in Table I.

electrochemical potential by the addition of ionophores led to about a twofold stimulation of the enzyme activity. This behavior is similar to other proton-translocating enzymes and can be explained by uncoupling the established Δ μH^+ from ATPase or PPase.

As can be seen in Figure 1 ATP-driven transport shows a distinct pH optimum at 7.3. This is in good agreement with data presented previously (16, 17). Pyrophosphate-stimulated transport exhibited a relative broad pH optimum with maximal rates at 7.8. As shown by Marquardt and Lüttge (15) for *Kalanchoe daigremontiana*, PPase-driven H^+ translocation at the tonoplast membrane showed maximal rates in the alkaline range at pH 8.2 to 8.7.

As shown in Figure 2, A and B, phenylalanine transport exhibited saturation kinetics with respect to ATP and PPi. Apparent K_m-values were determined by Lineweaver-Burk plots to be 0.5 mM for ATP and 0.25 mM for PPi. According to Stitt *et al.* (23), the level of ATP in the cytosol is in the range of 1.5 mM and would be sufficient to drive phenylalanine transport at near maximum rate. Smyth and Black (22) presented data that PPi level in pea and corn tissues are in the range of 5 to 39 nmol PPi per mg fresh weight. Assuming that all cellular PPi is located in the cytosol, Taiz (25) concluded that PPi level may reach 0.39 mM. In spinach, a cytosolic concentration of about 0.2 to 0.3 mM was found (28). Therefore, PPi levels also seem to be high enough to ensure PPase-driven phenylalanine uptake at near maximum rate. $K_{m\,app}$ values of ATPase- (Fig. 2C) and PPase-driven (Fig. 2D) phenylalanine transport are calculated at 1.2 to 1.4 mM which is in the range of internal vacuolar concentration (10). Maximal rates of transport were reached at 120 to 140 nmol L-Phe·mg $Chl^{-1}\cdot h^{-1}$, high enough to ensure that all synthesized phenylalanine is transported into the vacuole. In contrast to transport the involved H^+-translocating enzymes exhibited relatively broad pH optima in the more alkaline range (Fig. 3). Highest rates of ATP hydrolysis were obtained between pH 7.8 to 8.5 and for PPi hydrolysis between pH 8 to 9. Substrate hydrolysis followed Michaelis-Menten kinetics and half-saturation was reached at 0.45 mM ATP or 23 μM PPi (Fig. 4). Maximal rates were determined to be 5.95 μmol $ATP_{hydrolysed}$·mg $Chl^{-1}\cdot h^{-1}$ and 4.75 μmol $PPi_{hydrolysed}$·mg $Chl^{-1}\cdot h^{-1}$. The addition of 1.5 mM L-phenylalanine to the enzyme assay (data not shown) which may stimulate the rate of hydrolysis by a consumption of protons did not cause significant changes because phenylalanine transport is about 50-times slower than the apparent V_{max} of ATP or PPi hydrolysis. Table III shows the influence of other amino acids or amino acid analogs on rates of ATP- or PPi-stimulated phenylalanine transport. A fivefold surplus of L-Ala, L-Val, D-Leu as well as D-Phe did not cause any changes in L-phenylalanine uptake. L-Leu and L-Ile, more lipophilic amino acids (18 or "Materials and Methods"), markedly inhibited phenylalanine transport. Strongest decrease of transport was reached by the addition of L-Trp, the most lipophilic amino acid, while D-Trp was less effective. *P*-Fluorophenylalanine a substance known to inhibit amino acid uptake at the plasmalemma of *Lemna gibba* (12) also decreased the transport

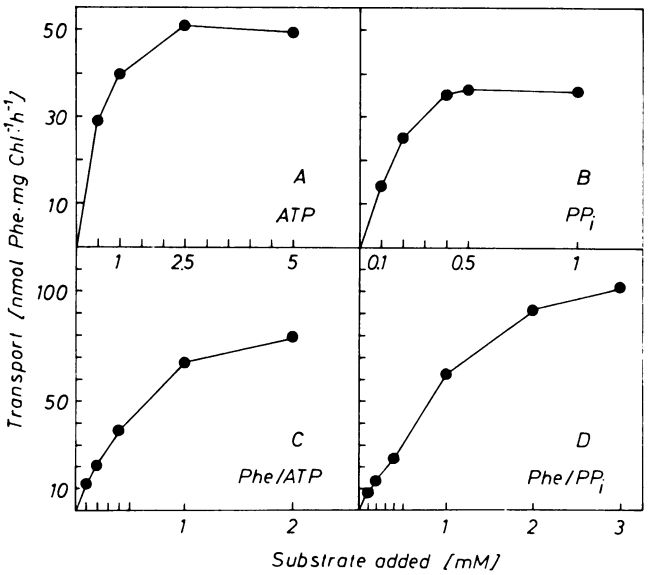

Figure 2. Transport of L-phenylalanine into isolated barley vacuoles as a function of ATP, PPi, and L-phenylalanine concentrations. A, Influence of ATP concentration. The assay medium (pH 7.4) additionally contained 1.5 mM L-phenylalanine; ATP was added as Mg-salt. B, Influence of PPi concentration. The assay medium (pH 7.8) additionally contained 1.5 mM L-phenylalanine and 40 mM K-gluconate. At each indicated PPi concentration, a 10-fold surplus of Mg^{2+} was added as gluconate. A and B have been corrected for basal nonactivated transport rate. C and D, Influence of L-phenylalanine concentration with regard to ATP- and PPi-driven transport. The assay medium additionally contained 5 mM MgATP (Fig. 2C) or 0.5 mM PPi, 5 mM Mg-gluconate, and 40 mM K-gluconate (Fig. 2D). The rates were calculated as described in Table I.

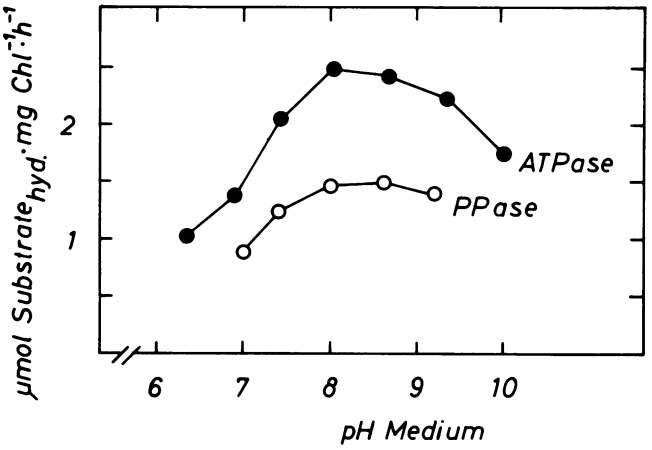

Figure 3. Direct measurement of ATPase and PPase as a function of the assay pH. ATP and PPi hydrolysis were measured as described in "Materials and Methods." The buffer capacity of the standard reaction media was increased to 50 mM. The following buffers were used: Mes for pH values between 6 to 7, Hepes for pH values between 7 to 8, and Tricine for pH values above 8. No significant changes in the rates of hydrolysis were observed when the buffers were changed (data not shown).

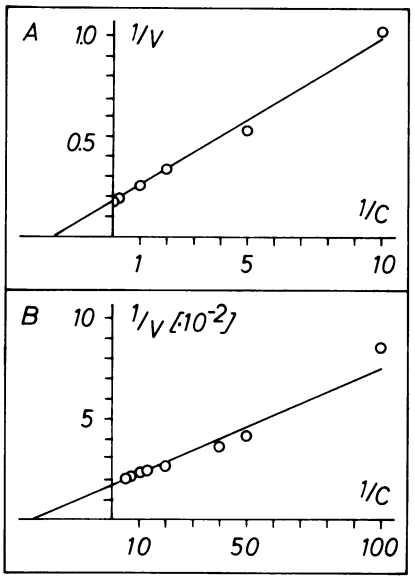

Figure 4. Lineweaver-Burk plots of ATPase (A) or PPase (B). Incubation and enzyme assay were done as described in "Materials and Methods." Apparent K_m-values were determined to 0.45 mM for ATP and 23 µM for PPi. $V_{max\ app}$ was (in µmol substrate$_{hydrolysed}$·mg Chl^{-1}·h^{-1}) 5.95 for ATPase and 4.95 for PPase.

Table III. *Influence of Several Amino Acids and Analogs on Rates of ATP- and PPi-driven L-Phenylalanine Transport*

The assay of ATPase-driven transport (pH 7.4) contained 5 mM MgATP, the assay for PPase-driven transport (pH 7.8) consisted of 0.5 mM PPi plus 5 mM Mg-gluconate and 40 mM K-gluconate. L-Phenylalanine (1.5 mM) was presumably present in all experiments and 100% expresses (in nmol L-Phe·mg Chl^{-1}·h^{-1}) 81.08 for ATPase and 56.09 for PPase.

Substrate	+ ATP	+ PPi
mM	%	%
L-Phe (1.5)	100	100
D-Phe (7.5)	100.6	97.1
L-Val (7.5)	96.6	96.7
L-Ala (7.5)	105.2	98.3
L-Leu (7.5)	47.8	62.2
L-Ile (7.5)	56.0	71.9
L-Trp (7.5)	27.0	32.6
D-Leu (7.5)	97.9	102.8
D-Trp (7.5)	42.5	55.0
p-F-Phe (7.5)	46.7	41.5
Phe-Pyr (7.5)	99.3	ND[a]

[a] Not determined.

rates. The addition of phenylpyruvate had no effect on phenylalanine uptake and it seems that the tonoplast carrier has a stereospecific affinity for L-amino acids. Additionally, the relative hydrophobicity of the transported substance is of great influence, therefore, also D-Trp is able to compete with phenylalanine uptake.

As shown in Table IV the uptake of L-Leu, L-Tyr, and L-Trp is also stimulated by the addition of ATP. A fivefold surplus of L-Phe lowered the rates of transport markedly (data not shown). This gives further support to the idea that trans-

Table IV. *MgATP-Dependent Transport of Aromatic Amino Acids and Leucine into Isolated Barley Vacuoles*

The assay medium (pH 7.4) contained 5 mM MgATP and 1.5 mM (0.65 mM for L-Tyr) of the aromatic amino acid; 100% expresses (in nmol amino acid·mg $Chl^{-1}\cdot h^{-1}$) 88.7 for L-Phe, 73.4 for L-Leu, 123 for L-Trp, and 37.8 for L-Tyr.

Substrate	Rate of Transport	
	+ MgATP	− MgATP
mM	%	
L-Phe (1.5)	100	41.1
L-Leu (1.5)	100	50.7
L-Trp (1.5)	100	44.9
L-Tyr (0.65)	100	55.1

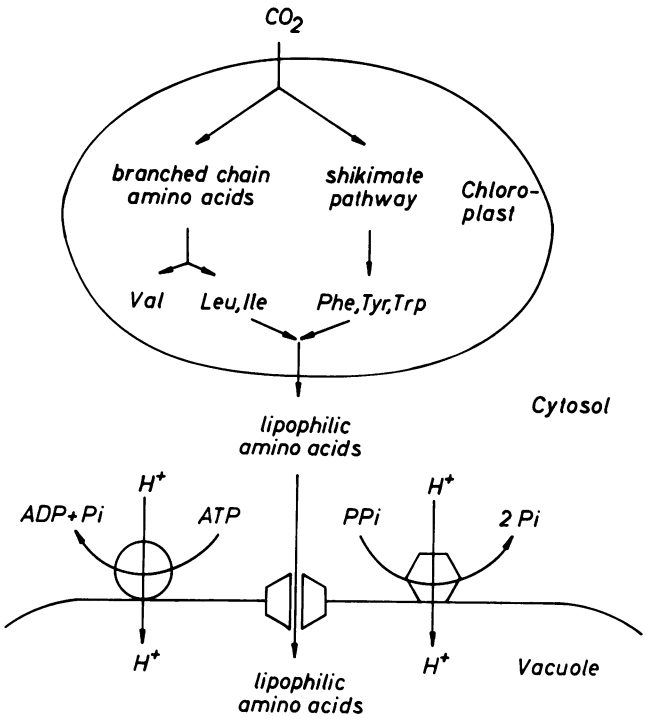

Figure 5. (Scheme) Formation and transport of hydrophobic L-amino acids in barley protoplasts. The transport into the vacuole is mediated by a so-called 'hydrophobic L-amino acid carrier' energized both by tonoplast ATPase and PPase.

port of lipophilic amino acids is mediated by the same carrier system.

DISCUSSION

As reviewed by Rea and Sanders (20), the tonoplast membrane possesses two distinct enzymes both able to translocate protons into the vacuolar compartment. The established electrochemical proton potential difference $\Delta\ \mu H^+$ is used as the driving force for phenylalanine transport into the vacuole. For the first time data are presented that this transport could be driven by either energy-supplying enzymes. The addition of ionophores markedly inhibited phenylalanine transport while rates of ATP and PPi hydrolysis were accellerated. ATP-driven transport shows a distinct pH optimum at 7.3 which is in good agreement with other investigators (16, 17) and seems to resemble cytosolic conditions. PPi-driven uptake of phenylalanine exhibits a relatively broad pH optimum with maximal rates at 7.8. However, at pH 7.3 the PPi-driven transport has nearly 95% of its maximal activity.

As shown, phenylalanine uptake obeys saturation kinetics for ATP and PPi as well as phenylalanine. Barley mesophyll protoplasts synthesize phenylalanine in the range of 15 nmol Phe·mg $Chl^{-1}\cdot h^{-1}$ (14). The high capacity of phenylalanine transport across the tonoplast membrane ($V_{max\ app}$ = 120–140 nmol Phe·mg $Chl^{-1}\cdot h^{-1}$) guarantees that all phenylalanine which is synthesized in the chloroplast and exported into the cytosol can be directly transferred into the vacuole. This agrees with the finding that up to 95% of the free cellular phenylalanine is found in this compartment (2; E Martinoia, personal communication). Direct measurement of the involved H^+-translocating enzymes revealed that both activities exhibited broad pH optima. This is in good agreement with data from Takeshige *et al.* (27). Both enzymes have maximal rates of about 4.75 (PPase) to 5.95 (ATPase) μmol $substrate_{hyd}$·mg $Chl^{-1}\cdot h^{-1}$, enough to maintain tonoplast energization during the transport process. Apparent K_m-values of 0.45 mM for ATP and 23 μM for PPi are in the range as described in (15).

The physiological role of the H^+-translocating PPase is not yet clear. PPi is generated with the formation of sucrose, starch, and cellulose and is used as a substrate for PPi-dependent phosphofructokinase (5). Therefore, as proposed by Rea and Sanders (20), the tonoplast-bound pyrophosphatase may, *in vivo*, work reversibly to stabilize PPi- or H^+-level in the cytosol.

The transport of L-phenylalanine is not affected by D-Phe or D-Leu. Jung and Lüttge (11) found that D- and L-Ala are both competing for the same carrier system at the plasmalemma in *Lemna gibba*. Higgins and Payne (9) were able to demonstrate that only L-isomers of Ala, Leu, and Val are transported across the membranes in germinating barley embryos. In our experiments, transport rates were unaffected by L-Ala and L-Val. With increasing hydrophobicity of the added amino acid a striking influence on phenylalanine uptake was noticed. L-Phenylalanine transport is strongly affected by L-Trp and less by D-Trp, L-Leu, and L-Ile. The low effect of D-Trp may indicate that the carrier system possesses a high affinity for the "right configuration." This agrees with the finding that D-Phe and D-Leu did not decrease L-phenylalanine transport at all. Both amino acids are of lower hydrophobicity than tryptophan (18 or "Materials and Methods"). Phenylpyruvate had no detectable influence on transport rates indicating that carrier mediated transport is coupled to the amino group of the amino acid. In yeast seven independent amino acid carriers have been found (21), and three distinct carrier systems with affinities for Phe-Trp, Tyr, and Leu-Ile were described. We suggest that for barley there might be only one carrier for the lipophilic amino acids.

Figure 5 presents an overview of phenylalanine synthesis and transport in barley protoplasts. Chloroplasts export their product into the cytosol from where it is transferred into the vacuole. This is mediated by a so-called hydrophobic amino acid carrier system. As claimed by Driessen *et al.* (6), this transport is of high affinity toward the substrate. The transport

operates by energization, at least *in vitro*, by both H^+-translocating enzymes, tonoplast ATPase and PPase. The specificity of the carrier system and its coupling to energy sources is under further investigation.

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