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Amino Acid Transport across the Tonoplast of Vacuoles Isolated from Barley Mesophyll Protoplasts[1]

Uptake of Alanine, Leucine, and Glutamine

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ABSTRACT

Mesophyll protoplasts from leaves of well-fertilized barley (*Hordeum vulgare* L.) plants contained amino acids at concentrations as high as 120 millimoles per liter. With the exception of glutamic acid, which is predominantly localized in the cytoplasm, a major part of all other amino acids was contained inside the large central vacuole. Alanine, leucine, and glutamine are the dominant vacuolar amino acids in barley. Their transport into isolated vacuoles was studied using ^{14}C-labeled amino acids. Uptake was slow in the absence of ATP. A three- to sixfold stimulation of uptake was observed after addition of ATP or adenylyl imidodiphosphate an ATP analogue not being hydrolyzed by ATPases. Other nucleotides were ineffective in increasing the rate of uptake. ATP-Stimulated amino acid transport was not dependent on the transtonoplast pH or membrane potential. *p*-Chloromercuriphenylsulfonic acid and *n*-ethyl maleimide increased transport independently of ATP. Neutral amino acids such as valine or leucine effectively decreased the rate of alanine transport. Glutamine and glycine were less effective or not effective as competitive inhibitors of alanine transport. The results indicate the existence of a uniport translocator specific for neutral or basic amino acids that is under control of metabolic effectors.

Within plant cells, sites of synthesis, storage, and degradation of amino acids and proteins have been studied in considerable detail. Different compartments such as chloroplast, cytosol, and vacuole are involved in amino acid metabolism and storage (20). Interaction of different compartments requires transport across membranes. Vacuoles function mainly in storage of amino acids. When synthesis exceeds demand by cytoplasmic protein synthesis and export from the cells, amino acids are transported into the vacuoles. At times of increasing demand, stored amino acids are mobilized and transported to the cytosol. This has been shown under conditions of sulfur starvation (5, 6) during which amino acids accumulate in the leaves. Stored amino acids are consumed when sulfur becomes available for growth.

Amino acid transport across plasma membranes has been studied in algae, oat coleoptiles, *Vicia*, *Commelina*, and *Lemna* leaves, suspension cultures, and cells isolated from various plant sources (2, 4, 14, 15, 18, 19). The results indicate the existence of different uptake systems for basic, neutral, and acidic amino acids. Uptake of amino acids across the plasmalemma is energized by the pmf.[3] However, little is known about transport across the tonoplast membrane of higher plants. Homeyer and Schultz (9) have investigated transport of phenylalanine into barley mesophyll vacuoles. Uptake was dependent on the pmf. Only in eucaryotic microorganisms active transport of amino acids into vacuoles has been studied in more detail (24; for review see ref. 10). Seven transport systems with high specificity were found to catalyze an nH^+amino acid antiport. Thus, amino acid uptake by yeast vacuoles also depended on the pmf which was sustained by the activity of an ATPase.

In the present paper, uptake of alanine, leucine, and glutamine was studied in isolated mesophyll vacuoles. The data suggest a transport mechanism that is different from that described for yeast vacuoles and for uptake of phenylalanine across the tonoplast of higher plants.

MATERIAL AND METHODS

Plant Growth

Barley (*Hordeum vulgare*, cv Gerbel) was grown in soil in a growth chamber; the light regime was 14 h light and 10 h dark; the temperature was 18°C in the dark and 20°C in the light. Primary leaves of 10 d old plants were harvested at the beginning of the light period.

Isolation of Vacuoles

Protoplasts and vacuoles were isolated as described by Martinoia *et al.* (16) and by Kaiser *et al.* (11). Vacuoles were liberated from the protoplasts by mechanical lysis. For this,

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[3] Abbreviations: pmf, proton motive force; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NEM, *N*-ethyl maleimide; PCMBS, *p*-chloromercuriphenylsulfonic acid.

the protoplasts were suspended in a solution containing 400 mmol L^{-1} sorbitol, 30 mmol L^{-1} Hepes-KOH (pH 7.8), 1 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} $CaCl_2$, 2 mmol L^{-1} EDTA, 30 mmol L^{-1} KCl, 0.2% (w/v) bovine serum albumin, and 20% Percoll. The suspension was forced through a needle (100 × 0.9 mm) which caused disruption of the plasmalemma. Liberated vacuoles were recovered by flotation by overlayering 10 mL of lysate with 8 mL of Percoll-free medium (as above) and with 2 mL of glycine betaine medium (medium as above: glycine betaine substituted for sorbitol). After centrifugation for 3 min at 100*g* and 10 min at 1200*g*, vacuoles were recovered at the interphase between sorbitol medium and glycine betaine medium. Mechanical lysis was repeated until all protoplasts had been ruptured. Vacuoles were pooled, concentrated by flotation, and transfered into a medium where 30 mmol L^{-1} K-gluconate replaced all chloride salts.

Transport Experiments

Uptake of amino acids by isolated vacuoles was measured at 20°C. To separate the vacuoles from the incubation medium, the silicon oil layer centrifugation technique previously described by Kaiser and Heber (12) and Martinoia *et al.* (17) was employed. For each condition and time point, five polypropylene microcentrifugation tubes (with a capacity of 400 μL) were prepared as follows: 40 μL of vacuole suspension were added to 60 μL of medium containing 67% Percoll, 0.35 mol L^{-1} sorbitol, 45 mmol L^{-1} potassium gluconate, 30 mmol L^{-1} Hepes-KOH (pH 7.0), 3.3 mmol L^{-1} DTT, 0.3% (w/v) purified bovine serum albumin, and other solutes as indicated. The samples were overlayered with 150 μL phenylmethyl silicone oil (AR 200, Wacker Chemie, München, FRG) and then 40 μL of H_2O. Amino acid uptake was terminated by centrifugation at 10,000*g* for 30 s. Intact vacuoles floated through the silicone layer into the aqueous phase, which was recovered and used for measurements. For the transport of labeled amino acids, each sample contained ^3H_2O and L-(U-^{14}C)alanine or L-(U-^{14}C)leucine or L-(U-^{14}C)glutamine at an activity of 5 to 6 kBq, ^3H_2O equilibrates rapidly between medium and vacuoles; the radioactivity of ^3H_2O in the upper phase after centrifugation was used to quantify the recovery of vacuoles (17).

Amino Acid Determination

Aliquots (80 μL) of the samples were added to 20 μL of 12.5% (w/v) 5-sulfosalicylic acid dihydrate, incubated at 0°C for 30 min, and centrifuged (10,000*g*, 10 min). The supernatant was diluted with buffer containing 9.4 g trilithium citrate-4-hydrate, 7.4*g* citric acid monohydrate, and 0.5% (v/v) 2,2′-thiodiethanol in 1 L.

Amino acid concentrations were determined with an amino acid analyzer (Biotronik, LC 5001, Maintal, FRG). The method was based on liquid ion exchange chromatography followed by detection with ninhydrin. Calculations of amino acid concentrations were based on the following relationships between volume and Chl: 1 mg Chl is contained in 10^7 protoplasts; 10^7 protoplasts have a volume of 200 μL and 10^7 vacuoles a volume of 160 μL; each protoplast contains one vacuole.

α-Mannosidase Determination

Two aliquots of 20 μL each were used to measure the activity of α-mannosidase. α-Mannosidase is exclusively located in the vacuole of barley mesophyll protoplasts and was used to quantify the recovery of vacuoles in the upper phase after silicon oil centrifugation and to compare amino acid contents of vacuoles and protoplasts (16).

RESULTS

Amino Acid Contents of Mesophyll Protoplasts and Vacuoles

Protoplasts and vacuoles were isolated from barley leaves and analyzed for amino acids. The total amino acid concentration in protoplasts was 120 ± 43 mmol L^{-1} and in isolated vacuoles was 77 ± 32 mmol L^{-1}. Part but not much of the vacuolar amino acids was lost during isolation (8). To make the relative distribution of amino acids in protoplasts and vacuoles comparable, amino acid contents were calculated not only as concentrations, but also as percent amino acid recovered in the vacuole and as mol % (Table I). Most amino acids were found in comparable molar ratios in vacuoles and protoplasts. However, the concentration of glutamic acid was much larger in protoplasts than in vacuoles indicating predominant localization outside the vacuole. The data suggest higher concentrations in the cytoplasm than in the vacuole. This is particularly true for glycine, lysine, threonine, and serine. In the following, transport of the dominant vacuolar amino acids, alanine, glutamine, and leucine, across the tonoplast will be investigated.

Uptake of ^{14}C-Labeled Amino Acids by Isolated Vacuoles

Figure 1A shows the time-dependent uptake of [^{14}C]alanine by isolated vacuoles. Vacuoles were incubated in a medium containing 0.1, 0.5, and 1 mmol L^{-1} alanine. Uptake was linear with time between 4 and 20 min of incubation. The interceptions of the regression lines with the ordinate reflect adsorption of [^{14}C]alanine to the tonoplast and carry over of [^{14}C]alanine from the incubation medium through the silicone oil layer into the upper aqueous phase. From the slopes, rates of uptake were computed. Uptake increased with concentration (Fig. 1B).

Effect of ATP on Uptake of [^{14}C]Alanine

When MgATP or ATP was added to the incubation medium, the uptake of alanine by vacuoles increased considerably (Fig. 2). Averaged over 12 experiments, the uptake rate in the presence of 2 mmol L^{-1} alanine was 0.90 ± 0.45 nmol 10^{-7} vacuoles min^{-1} without ATP and 5.05 ± 1.77 nmol 10^{-7} vacuoles min^{-1} with MgATP. Interestingly, ATP was almost as effective as MgATP in stimulating uptake. EDTA at a concentration of 2 mmol L^{-1} was added to the incubation medium to exclude the possibility of free Mg^{2+} either from the isolation procedure or from breakage of vacuoles during the time course of the experiment. Still, ATP (90%) stimulated uptake of alanine almost as effectively as MgATP (100%). H^+-ATPases are dependent on MgATP as substrate (22).

Table I. *Amino Acid Concentrations in Protoplasts and Vacuoles Isolated from Barley Primary Leaves*

The calculations of concentrations were based on a volume of 200 μL per 10^7 protoplasts and of 160 μL per 10^7 vacuoles. To compare the relative distribution of amino acids in protoplasts and vacuoles, amino acid contents are also given in mol%, *i.e.* as ratio of specific to total amino acid concentration. (number of experiments: 4; mean values ± SD).

Amino Acid	Protoplasts	Vacuoles	% in Vacuoles	Protoplasts	Vacuoles
	mmol L^{-1}			*(mol/mol)*	
Alanine	29.8 ± 5.1[a]	19.2 ± 4.4	52	24.7 ± 5.4	25.0 ± 7.1
Arginine	6.2 ± 2.4	3.8 ± 2.0	49	5.1 ± 2.1	5.0 ± 2.6
Asparagine	3.5 ± 2.8	2.5 ± 1.9	57	2.9 ± 1.3	3.2 ± 0.9
Aspartic acid	0.4 ± 0.1	0.2 ± 0.1	40	0.3 ± 0.1	0.3 ± 0.1
Glutamic acid	3.6 ± 0.9	0.2 ± 0.2	4	3.0 ± 1.1	0.3 ± 0.3
Glutamine	22.7 ± 12.0	15.3 ± 14.2	54	18.8 ± 5.2	19.9 ± 9.3
Glycine	1.0 ± 0.7	0.4 ± 0.2	32	0.8 ± 0.3	0.5 ± 0.3
Histidine	2.0 ± 1.1	1.2 ± 0.7	48	1.7 ± 0.3	1.6 ± 0.2
Isoleucine	3.3 ± 1.7	2.7 ± 1.3	65	2.7 ± 0.4	3.5 ± 0.3
Leucine	11.4 ± 4.5	9.4 ± 3.7	66	9.5 ± 1.4	12.3 ± 2.5
Lysine	8.0 ± 4.8	2.4 ± 1.8	24	6.6 ± 1.6	3.1 ± 1.6
Methionine	1.7 ± 0.9	1.2 ± 0.5	56	1.4 ± 0.3	1.6 ± 0.3
Phenylalanine	5.8 ± 2.1	4.4 ± 1.6	61	4.8 ± 0.6	5.7 ± 1.1
Serine	4.5 ± 2.5	2.3 ± 1.0	41	3.7 ± 0.8	3.0 ± 0.6
Threonine	4.6 ± 0.8	2.3 ± 1.1	40	3.8 ± 0.5	3.0 ± 0.3
Tyrosine	4.2 ± 1.3	3.4 ± 1.2	65	3.5 ± 0.5	4.4 ± 0.8
Valine	7.9 ± 3.5	5.8 ± 2.5	59	6.6 ± 1.1	7.6 ± 1.3

[a] Mean ± SD, $n = 4$.

Therefore, the result is a first indication that ATP stimulation of alanine uptake does not depend on ATP hydrolysis.

Stimulation of alanine uptake by ATP was saturated with 10 mmol L^{-1} ATP in the incubation medium (Fig. 3). Half-saturation with ATP was observed between 1 and 2 mmol L^{-1}. It should be mentioned that the stimulatory effect of ATP was somewhat lower in these experiments than is usually observed.

Specificity of Stimulation of [^{14}C]Alanine Uptake

Table II shows that [^{14}C]alanine uptake could not be stimulated by the addition of GTP, ADP, or AMP. However, the ATP analog AMPPNP was as effective as ATP in increasing transport of alanine. AMPPNP cannot be cleaved by ATPases. Obviously, binding of ATP and ATP analogs activated amino acid transport. Phosphate and pyrophosphate could not stimulate uptake of [^{14}C]alanine. Thus, the binding site for stimulation is specific for ATP and the ATP-analog AMP PNP.

Effects of ATPase-Inhibitors, Protein Modifiers, and the Role of Membrane Potential and Transtonoplast pH

Although the stimulatory effect of AMPPNP suggests that the tonoplast ATPase is not directly involved in stimulating alanine import, experiments were conducted to characterize the dependence of alanine uptake on the transtonoplast membrane potential and pH. Nitrate, which is an inhibitor of the tonoplast ATPase, did not decrease the uptake rate of [^{14}C] alanine in the presence of ATP (data not shown). PCMBS and NEM, which modify SH-groups of polypeptides, increased the rate of [^{14}C]alanine uptake without added ATP. To some extent, stimulation by PCMBS or NEM was additive with respect to the stimulation by ATP (Table III). Therefore, SH-groups may be involved in the regulation of [^{14}C]alanine uptake.

The results provide strong evidence that a proton-translocating ATPase is not directly involved in alanine transport. However, an electrochemical gradient such as the proton motive force could be involved in amino acid transport. Table IV summarizes the effects of reagents which influence the pmf of membranes on [^{14}C]alanine uptake. The potassium ionophor valinomycin decreases the electrical component of the pmf by allowing free diffusion of K^+. Nigericin catalyzes a H^+/K^+-exchange and CCCP is a protonophor. NH_4Cl also decreases the pH across the tonoplast membrane. None of these compounds inhibited alanine transport into the vacuoles. Isolated vacuoles as used in these experiments rapidly accumulate neutral red (microscopic analysis, result not shown). This indicates a pH gradient across the tonoplast membrane. We conclude that alanine transport into isolated vacuoles is independent of the pmf and therefore is not coupled to the primary energization by the tonoplast H^+-ATPase.

Inhibition of Alanine Uptake by Other Amino Acids

Table V shows competition between alanine and other amino acids for the transport system. Glycine and glutamine were not effective in inhibiting uptake of [^{14}C]alanine, whereas valine, leucine, and methionine (the latter not shown) considerably decreased ATP-stimulated uptake rates. In the absence

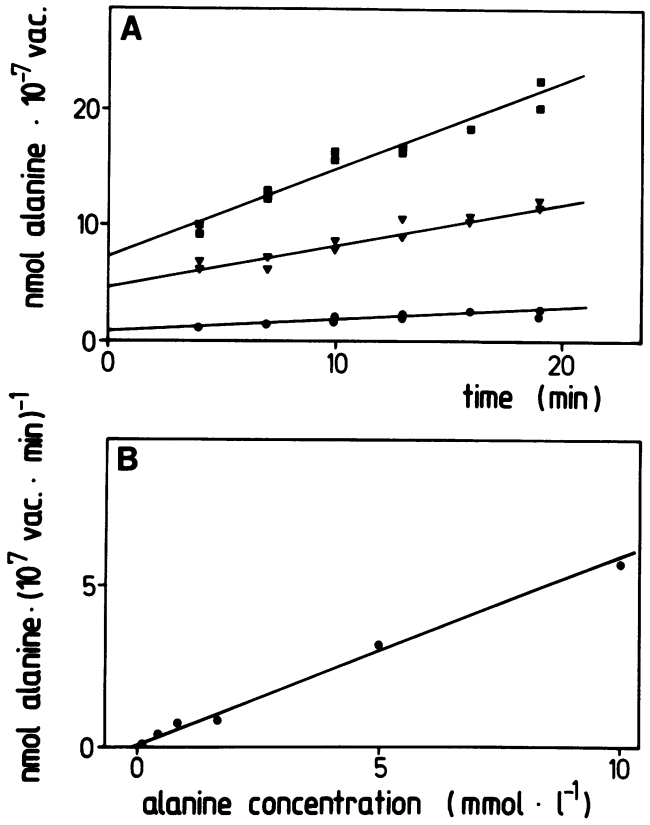

Figure 1. A, Uptake of [^{14}C]alanine by isolated barley mesophyll vacuoles as a function of time. Vacuoles were incubated in the presence of 0.1 (●), 0.5 (▼), and 1 mmol L^{-1} (■) alanine. At the times indicated, they were flotated through a silicon oil layer and counted for ^{3}H- and ^{14}C-radioactivity. For more information see text. B, Concentration dependence of alanine uptake. Uptake rates were determined by regression analysis of slopes of uptake kinetics as shown in (A). Any point is derived from six measurements. The data were obtained from two experiments with different plant material. No ATP was added.

of ATP, the low uptake rates were not further decreased by the addition of neutral amino acids.

Uptake of Glutamine, Leucine, and Methionine by Isolated Vacuoles

Glutamine transport into vacuoles had similar characteristics as transport of alanine. Uptake of glutamine was linear with incubation time. In the absence of ATP, uptake of glutamine also increased linearly with concentration (data not shown). Uptake in the presence of 2 mmol L^{-1} glutamine was 0.94 ± 0.49 without ATP and 3.86 ± 1.88 (n = 5) with MgATP. Uptake of glutamine was stimulated by PCMBS, both in the presence and in the absence of ATP. CCCP, nitrate, and NH_4Cl did not inhibit glutamine transport (Table VI).

At comparable concentrations, rates of leucine uptake were lower than rates of uptake of glutamine or alanine. Stimulation by ATP was less than threefold. Lowest uptake rates were observed for methionine (Fig. 4). Uptake of leucine in the

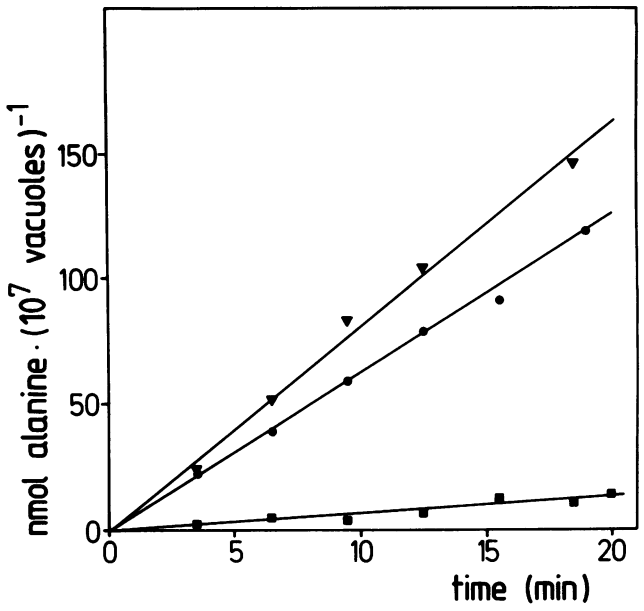

Figure 2. Effect of ATP and MgATP on time-dependent uptake of [^{14}C]alanine by isolated vacuoles. ATP (●) and MgATP (▼) were added at a concentration of 10 mmol L^{-1}. As a control (■), alanine uptake also was measured in the absence of MgATP. The alanine concentration was 2 mmol L^{-1}.

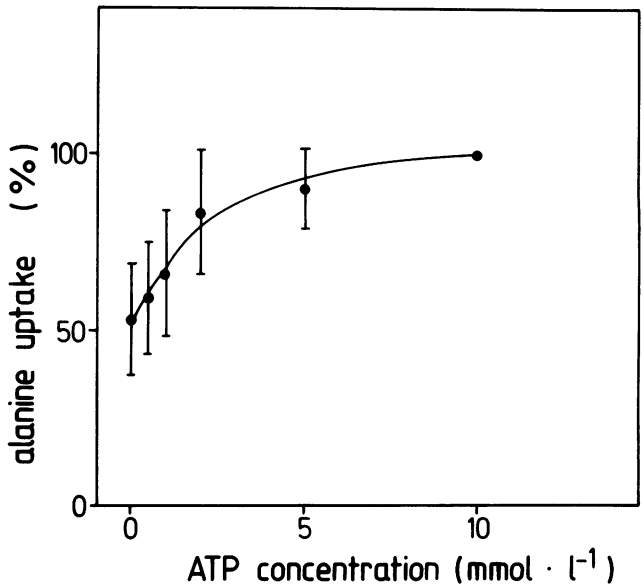

Figure 3. Rate of [^{14}C]alanine uptake in dependence of the MgATP concentration. Alanine was added at a concentration of 2 mmol L^{-1} (100% corresponds to 1.7 nmol [10^7 vacuoles min]$^{-1}$). The points are mean values of four experiments. In two of these experiments, ammonium molybdate was added at a final concentration of 0.1 mmol L^{-1} to prevent ATP hydrolysis by acid phosphatases which are released from broken vacuoles. Both assays gave similar results.

absence of ATP was linearly dependent on concentration. In the presence of ATP, methionine uptake revealed a saturable component at low concentrations (≤1 mmol L^{-1}) (7) and a linear increase at high methionine concentrations. Uptake of

Table II. *Effects of GTP, ADP, AMP, AMPPNP, PP_i, and P_i on Uptake of [^{14}C]-Alanine by Isolated Vacuoles*

Each uptake value is derived from the kinetics of six time points. Uptake was calculated by linear regression analysis. The alanine concentration was 2 mmol L^{-1}.

Effector	Concentration	Uptake Rate	Stimulation
	mmol L^{-1}	*nmol Ala (10^7 vacuoles min)$^{-1}$*	%
Experiment 1			
None		0.57	0
MgATP	5	2.70	100
AMP	5	0.64	3
$MgPP_i$	0.1	0.89	15
P_i	5	0.57	0
Experiment 2			
none		0.71	0
MgATP	10	8.1	100
MgGTP	10	0.97	3
MgADP	10	0.87	2
Experiment 3			
none		1.9	0
MgATP	10	5.4	100
AMPPNP+Mg^{2+}	10	5.3	97
AMPPNP/MgATP	10/10	5.1	91

Table III. *Effect of PCMBS and NEM on Uptake of [^{14}C]Alanine by Isolated Vacuoles*

For these experiments, the isolated vacuoles were transferred to BSA- and DTT-free solutions. The alanine concentration was 2 mmol L^{-1}.

Effector	Concentration	Uptake Rate	Stimulation
	mmol L^{-1}	*nmol Ala (10^7 vacuoles min)$^{-1}$*	%
Effect of NEM			
None		0.47	0
MgATP	10	2.6	100
NEM	2.5	2.1	77
NEM +	2.5+	4.2	175
MgATP	10		
Effect of PCMBS			
None		1.0	0
MgATP	10	4.9	100
PCMBS	1	8.9	202
PCMBS+	1+	9.6	221
MgATP	10		

leucine, glutamine, and alanine showed saturation at high concentrations.

Net Uptake of Amino Acids

Uptake of radiolabeled amino acids does not allow distinguishing between net uptake of amino acids and amino acid exchange between medium and vacuoles. Therefore, we investigated whether the transport of amino acids led to an increase in amino acid concentration. Isolated vacuoles were incubated in the presence of 10 mmol L^{-1} leucine for 2 and 20 min in the presence of 10 mmol L^{-1} ATP. Vacuoles were recovered by the silicon oil layer centrifugation and analyzed for leucine content. The leucine concentration increased from 6.6 ± 3.2 to 8.3 ± 4.0 mmol L^{-1} (n = 4). The initial concentration without external leucine added was 4.8 ± 3.0 mmol L^{-1}. The time-dependent change in vacuolar leucine concentration indicates an uptake rate of 15 nmol (10^7 vacuoles min)$^{-1}$. The 2-min value was needed to correct for carryover from the incubation medium through the silicon

Table IV. *Effect of Ionophores on ATP-Stimulated Alanine Transport*

The experiments were performed in standard incubation medium which contains K^+ at a concentration of 30 mmol L^{-1}. The alanine concentration was 2 mmol L^{-1}.

Effector	Concentration	Uptake Rate	Stimulation
	mmol L^{-1}	*nmol Ala (10^7 vacuoles min)$^{-1}$*	%
None		0.48	0
MgATP	10	6.2	100
NH_4Cl +	5+		
MgATP	10	6.5	105
Valinomycin +	10^{-2}+		
MgATP	10	6.3	102
Nigericin +	10^{-2}+		
MgATP	10	6.5	105
Valinomycin +	10^{-2}+		
CCCP	$4\cdot10^{-2}$+		
MgATP	10	5.9	95

Table V. *Inhibition of Alanine Transport by Other Amino Acids*

Vacuoles were incubated with 1 mmol L^{-1} alanine. Glutamine, glycine, leucine, and valine were added at a concentration of 30 mmol L^{-1}. MgATP was added at a concentration of 10 mmol L^{-1} (n = 10).

Added Amino Acid	Uptake Rate		
	% of control	*nmol (10^7 vacuoles min)$^{-1}$*	
	+ATP	+ATP	−ATP
Alanine	100	3.4	0.5
Glutamine	97	3.3	0.6
Glycine	94	3.2	0.4
Leucine	13	0.4	0.6
Valine	15	0.5	0.6

Table VI. *Uptake of [^{14}C]Glutamine by Isolated Mesophyll Vacuoles as Affected by Various Treatments*

The glutamine concentration was 2 mmol L^{-1}. See also legend to Table II.

Effector	Concentration	Uptake Rate	Stimulation
	mmol L^{-1}	*nmol (10^7 vacuoles min)$^{-1}$*	%
None		1.4	0
MgATP	10	2.6	100
PCMBS	1	2.6	100
PCMBS+	10+		
MgATP	1	4.0	217
MgATP	10	4.1	
NH_4Cl	1+		
MgATP	10	3.9	
KNO_3+	50+		
MgATP	10	4.0	
CCCP+	$4\cdot10^{-2}$+		
MgATP	10	4.0	

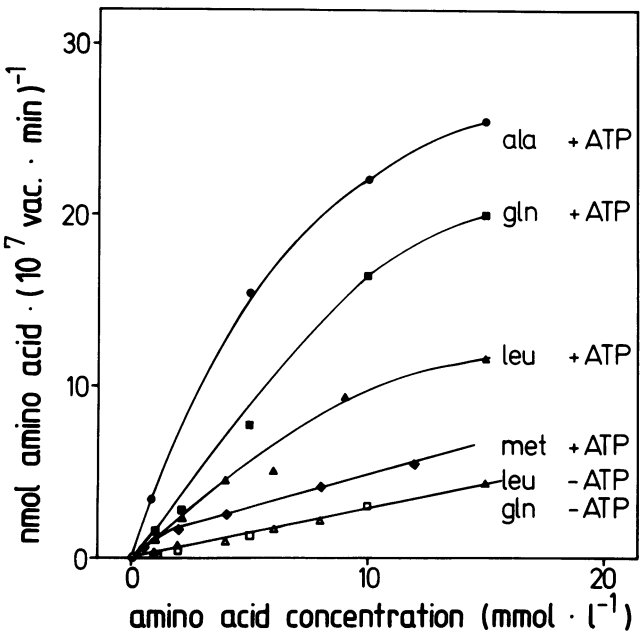

Figure 4. Uptake of [^{14}C]alanine, [^{14}C]glutamine, [^{14}C]leucine, and [^{35}S]methionine by isolated vacuoles as a function of concentration. Vacuoles were incubated with amino acids at varying concentrations in the presence (●, ■, ▲, ♦) or absence of MgATP (□, △). From time-dependent uptake kinetics as shown in Figure 1, uptake rates were computed.

oil layer into the upper aqueous medium and for adsorption of amino acids to the tonoplast membrane. Both effects have to be distinguished from uptake into the vacuoles. The results do not distinguish uniport transport of leucine from exchange of leucine against other endogenous amino acids. However, efflux experiments show that there is no one-to-one stochiometry between uptake and release of amino acids from the vacuole (8).

DISCUSSION

When barley plants are grown in soil or hydroponic culture, leaves have an osmotic potential of approximately 290 mosmol L^{-1} at the end of the dark period. Of this, 40 to 80 mosmol have to be attributed to amino acids. Compared to barley leaves, the concentrations of amino acids in protoplasts was increased.

This is mainly due to osmotic shrinkage during protoplast preparation. The incubation and suspension media have an osmolarity of close to 550 mosmol. Since the cytoplasm contributes only 20% of the total volume of mesophyll cells, a large fraction of the amino acids must be compartmentalized in the vacuole. During the light period, amino acids are synthesized in the chloroplast. Rapid appearance of newly synthesized amino acids in the vacuoles has been demonstrated by Kaiser *et al.* (11). And indeed, vacuoles contain large concentrations of amino acids. Our results show that the vacuolar amino acid composition is very similar to that of protoplasts. Considerable portions of cellular amino acids have also been localized inside the vacuoles by Wagner (25) and Alibert *et al.* (1). These authors found alanine, glutamine, and leucine among the most abundant amino acids in protoplasts and vacuoles either isolated from suspension cultured *Acer* cells or from *Tulipa* leaf cells. Our data suggest higher amino acid concentrations in the cytoplasm than in the vacuole. However, it is unlikely that the cytoplasmic amino acid concentration is close to 300 mmol L^{-1} as calculated under the assumption that vacuoles comprise 80% of the protoplast volume. Three explanations are possible: (a) Amino acids were lost during isolation of the vacuoles. (b) The assumed volume of the vacuoles is too large. (c) Amino acid content of the cytoplasm is increased by binding of amino acids and amino acids are further compartmentalized inside the cytoplasm.

As a mechanism of amino acid transport, diffusion through the tonoplast membrane does not allow sufficient rates of uptake for most amino acids detected inside the vacuoles. At the pH of the medium and of the cytosol (pH 7.2–7.6), the α-carboxyl groups (pK between 1.7 for cysteine and 2.6 for thyrosine) are almost completely dissociated, and the α-amino groups (pK between 8.3 for cysteine and 10.4 for threonine) and the amino groups of the side chains of lysine (pK = 10.5) and of arginine (pK = 12.5) are protonated. With two to three charged side groups, molecules of a relative molecular mass between 75 and 204 are only slightly permeable in lipid membranes. Transport systems must be present in the tonoplast membrane which catalyze the transport. The data of this communication characterize an amino acid transporter with unique features.

In contrast to amino acid transporters of the plasmalemma (for review see ref. 23) and of the tonoplast of microorganisms (24), the carrier in barley mesophyll cells does not depend on the pmf. Although ATP is not required as substrate, ATP and the ATP analog AMPPNP activate the transporter. The transmembrane pH and the membrane potential may be dissipated without inhibition of alanine and glutamine uptake. Regulation of transporter proteins by ATP-binding has been described for channels in plant and animal cells (3, 13, 21).

The affinity of the transporter for its substrate amino acids is low. Saturation was not observed up to a concentration of 20 mmol L^{-1}. At low substrate concentrations, uptake increased almost linearly with concentration. The highest rates were measured with alanine as substrate. Glutamine, leucine, and methionine were transported at lower rates. Interestingly, the differences in uptake rate corresponded to the changes in relative abundance of the amino acids inside the vacuoles: the highest amino acid concentration and the highest uptake rate were observed for alanine, followed by glutamine, leucine, and methionine. The increased uptake rate could therefore be explained partly by dilution of the transported radiolabeled amino acids into vacuolar amino acid pools of varying size. If uptake of amino acids from the medium and efflux of endogenous amino acids occur simultaneously, uptake rates of amino acids, which are present at high vacuolar concentrations, appear to be higher than uptake of amino acids with low vacuolar concentrations. However, the dilution effect is not sufficient to explain the observed differences.

Efflux of amino acids from isolated vacuoles was also activated by ATP. ATP-stimulated efflux was inhibited by

neutral amino acids. Leucine was more effective than alanine in decreasing amino acid efflux (8). Leucine at a concentration of 10 mmol L^{-1} decreased ATP-stimulated amino acid efflux to less than 10%. In this concentration range, uptake of leucine still responded linearly to an increase in leucine concentration. No satisfactory explanations are at hand to reconcile these observations. Although the similarity of ATP-stimulation suggests identity of the transporters that catalyze the uptake and efflux reactions, strong evidence is still not available. For this, the regulatory properties indicate an asymmetric structure of the transporter molecule. The adenylate-binding site and the efflux regulating amino acid binding site are exposed to the cytosol. There is no evidence for a corresponding influx regulating amino acid binding site inside the vacuole. Even in the presence of very high concentrations of amino acids inside the vacuole, uptake from the cytosol or medium into the vacuole is not inhibited. This kind of regulation seems to be useful; it allows amino acid uptake when cytosolic amino acids concentrations are high; only when cytosolic amino acid concentrations decrease to low levels is amino acid efflux catalyzed. Thus, storage of amino acids in the vacuole is regulated on a source/sink relationship. The balance between synthesis and utilization of amino acids in protein synthesis determines the vacuolar amino acid pools.

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