Mechanism of Amino Acid Uptake by Sugarcane Suspension Cells

ROGER E. WYSE* AND EWALD KOMOR
USDA-ARS, Crops Research Laboratory, Utah State University, Logan, Utah 84322 (R.E.W.); and Botanisches Institut Der Universitat Bayreuth, Lehrstuhl Pflanzenphysiologie, Universitatstrasse 30, D-8580 Bayreuth, West Germany (E.K.)

ABSTRACT

The amino acid carriers in sugarcane suspension cells were characterized for amino acid specificity and the stoichiometry of proton and potassium flux during amino acid transport.

Amino acid transport by sugarcane cells is dependent upon three distinct transport systems. One system is specific for neutral amino acids and transports all neutral amino acids including glutamine, asparagine, and histidine. The uptake of neutral amino acids is coupled to the uptake of one proton per amino acid; one potassium ion leaves the cells for charge compensation. Histidine is only taken up in the neutral form so that deprotonation of the charged imidazole nitrogen has to occur prior to uptake. The basic amino acids are transported by another system as uniport with charge-compensating efflux of protons and potassium. The acidic amino acids are transported by a third system. Acidic amino acids bind to the transport site only if the distal carboxyl group is in the dissociated form (i.e., if the acidic amino acid is anionic). Two protons are withdrawn from the medium and one potassium leaves the cell for charge compensation during the uptake of acidic amino acids. Common to all three uptake systems is a monovalent positively charged amino acid-proton carrier complex at the transport site.

Sugarcane storage parenchyma cells can be grown in cell suspension in inorganic salt medium supplemented with sucrose and a mixture of amino acids. The amino acids have a profound effect on cell growth and are used more rapidly than nitrate. This suggests one or more specific transport systems for amino acids. A transport system for arginine has been described in sugarcane suspension cells. The system was found to be very specific for the charged groups on the molecule, so that neutral arginine analogs such as citrullin or neutral canavanine did not compete with arginine. The uptake of neutral and acidic amino acids from the culture medium might proceed via the action of separate amino acid transport systems; however, there is no further conclusive evidence available despite the large amount of data. In soybean root cells, three different uptake systems were postulated, though inhibition occurred between all L-amino acids. In tobacco suspension cultures, one general carrier is postulated. From electrophysiological work on oat coleoptiles, three systems, one for neutral, one for acidic, and one for basic amino acids, were suggested; different proton-amino acid stoichiometries and uptake rates led to the same suggestion for castor bean cotyledons. One carrier for at least all neutral amino acids was postulated in *Lemna* (6) and *Riccia* (4). In these systems, even ammonium and other positively charged amines competed with the neutral amino acids. There are many reports where just one or two amino acids were tested without further classification of transport system specificity. Therefore, no generalization can be made on uptake of the protein amino acids.

Since considerable previous work on transport in sugarcane cells was available, we decided to characterize the amino acid transport system. This was also important for the understanding of the differential deprivation of nitrogenous compounds from the sugarcane culture medium. The mechanism of amino acid uptake had been suggested to be coupled to inflow of positive charges, since a specific depolarization of the membrane potential had been found. In castor bean cotyledons, net proton uptake was observed with amino acid uptake, but the stoichiometries were low—namely, 0.3, presumably due to the existence of diffusion barriers in the cotyledons. The use of suspension cells makes pH measurements much easier, and H+ stoichiometries are expected which better reflect the binding stoichiometry at the transport site. Since sugarcane cells are rigid and their transport properties are not damaged by conventional manipulation procedures, they are a suitable experimental system for transport studies.

MATERIALS AND METHODS

**Cell Culture.** The suspension culture of sugarcane was grown in White's basal salt medium supplemented with vitamins, sucrose, and yeast extract. The cells were used after 7 to 9 d of growth and transferred to basal salt medium the night before the experiment. The media was changed by decanting the supernatant twice. The starved cells were collected on a filter, washed three times with uptake media, weighed, and resuspended in uptake media to the desired concentration.

**Amino Acid Uptake.** The uptake experiments were performed with 0.5 g cells (fresh weight) in 5 ml of 5 mM sodium phosphate buffer pH 6.0. The amino acids were usually added to a 50 µM final concentration with a specific radioactivity of 1 kBg/mol. All amino acids used were 3H-labeled except for tryptophane, which was 14C-labeled. (All radiochemicals were from New England Nuclear.) One-ml samples were withdrawn at 1-min intervals, rapidly filtered, and washed with ice-cold buffer on filter paper. The maximum duration of an uptake experiment was 4 min. The filter with the cells was counted in scintillation solution (dioxan:naphthale:n-propanol(1:22:3) by scintillation spectrometry.

The *K*, and *V* values for amino acid uptake were determined by usual concentration dependence studies but the clas-
sification of amino acids into uptake systems was performed by inhibition experiments. The uptake of 50 μM labeled amino acid was measured in both the absence and presence of a 500 μM unlabeled amino acid. Appreciable inhibition (i.e. more than 20%) of labeled amino acid uptake by the competing amino acid was taken as evidence that the two respective amino acids shared the same uptake system. In several cases reciprocal inhibition experiments were conducted to confirm that two amino acids inhibited uptake in a quantitatively predicted way. Reciprocal inhibition was especially important in cases where inhibition of uptake of one amino acid by another was poor. Such a result could mean that either two different uptake systems were involved or the affinity of the 'inhibiting' amino acid was low. In the latter case, strong inhibition of uptake would be expected in the reciprocal experiment.

Ion Flux. Ion fluxes were measured in a stirred chamber with 0.3 g cells (fresh weight) in 3 ml of distilled H2O with a pH-electrode (Ingold, Zurich) and K+-electrode (Phillips, Eindhoven) which were connected to an amplifier and strip chart recorder (10). Cells were added to the chamber and the pH allowed to stabilize before amino acid addition. Stabilized pH values ranged from 5.5 to 6.0. The amino acids were added to final concentrations of 300 μM and 1 mM. The stoichiometries of H+ and K+ movement due to amino acid uptake were calculated from the initial slopes of H+ and K+ movement after calibrating each uptake suspension by addition of 10 mol of HCl and 100 nmol of KCl solutions. When stoichiometries of amino acids and ion flux were determined, the amino acid uptake rate was measured as described above simultaneous with ion flux.

All experiments were repeated twice and each treatment within an experiment replicated four times.

RESULTS

The classification of amino acid transport systems is complicated by the fact that high concentrations of amino acids with slightly hydrophobic side chains can inhibit energy-dependent transport processes, such as sugar transport (Cho et al., in preparation). Therefore inhibition studies must be performed at low concentrations of amino acids (e.g. mm or less).

In Table I, the results of a screening experiment are shown. In this experiment, the uptake of a labeled amino acid was determined in the presence of neutral acidic and basic amino acids. The uptake of labeled 50 μM alanine is inhibited in the presence of 500 μM of a series of other neutral amino acids. It was not important whether the neutral amino acids were alphabetic (leucine), aromatic (phenylalanine), with hydroxy- or mercaptogroups (serine, cysteine) or amides (glutamate). Therefore, it appears that all neutral amino acids share the same uptake system. Only marginally inhibitory for alanine uptake was histidine, but histidine uptake was strongly inhibited by alanine; therefore, histidine also belongs to this group. Clearly not inhibitory were arginine and glutamic acid. The results of a series of similar experiments indicated that the neutral amino acids shared one uptake system: threonine uptake was inhibited by serine, alanine, or methionine; glutamic uptake by alanine; methionine uptake by alanine and cysteine; asparagine uptake by glutamine; etc.

In these experiments, glutamic acid and arginine did not inhibit glutamine or histidine uptake; therefore, glutamic acid seems to belong to another uptake system, which does not accept alanine or arginine, but accepts aspartic acid. Arginine uptake was inhibited by lysine but not glutamine or alanine (Table II).

Measurements of the K_m and V_max values for the amino acids allowed a ranking of the amino acids according to their apparent carrier affinities (Table III).

The results of these experiments suggest the existence of three amino acid uptake systems. One uptake system is specific for all neutral amino acids, including the alphabetic amino acids (alanine, serine, leucine, etc.), the aromatic amino acids (phenylalanine, etc.), the amides (glutamine and asparagine), and histidine. Histidine has a pK of 6.0 and, therefore, is 50% positively charged at pH 6.0. It was surprising, therefore, that histidine is a substrate for the neutral amino acid uptake system, but not for the basic amino acid system. The basic amino acid uptake system only transported arginine and lysine, while the acidic amino acids were taken up by a third system, specific for glutamic and aspartic

<table>
<thead>
<tr>
<th>Labeled Amino Acid</th>
<th>Nonlabeled Amino Acid</th>
<th>Per cent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>Pro</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Gly</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Leu</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Phe</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Ser</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Thr</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Cys</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Met</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Phe</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Trp</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Tyr</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Leu</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Ile</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Val</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Asn</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Gln</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Ser</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Thr</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>His</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Arg</td>
<td>94</td>
</tr>
</tbody>
</table>
The uptake of labeled amino acids was tested at 50 μM in absence or presence of 500 μM nonlabeled amino acid.

<table>
<thead>
<tr>
<th>Labeled Amino Acid</th>
<th>Nonlabeled Amino Acid</th>
<th>Per cent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>Ala</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Gin</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>His</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Lys</td>
<td>98</td>
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<tr>
<td>Glu</td>
<td>Arg</td>
<td>100</td>
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<tr>
<td></td>
<td>Ala</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Asp</td>
<td>68</td>
</tr>
</tbody>
</table>

It has been found that transport of glycine depolarizes the membrane of sugarcane cells (5); therefore, the proton influx and potassium efflux are not directly coupled by an exchange carrier. The effect of histidine uptake on proton flux (Fig. 1) is unusual. Obviously, there is only a small proton uptake with a stoichiometry of 0.3, whereas the potassium efflux has a stoichiometry of near one (Table IV). This stoichiometry would be expected, however, if histidine was only taken up by the neutral amino acid transport system; therefore, only the neutral form would be expected to be bound to the transport site. Removal of neutral histidine would cause a shift in the dissociation equilibrium of histidine, releasing protons to the medium. For instance, at pH 5.7 (Fig. 1), 0.7 protons should be set free for each histidine molecule taken up (the pK of the imidazol-proton is 6.0). If the uptake of neutral histidine is coupled to uptake of one proton, then only the difference between the proton released in the medium and that taken up is observed, i.e. 0.3. Since for each histidine molecule transported, one positive charge transverses the membrane, one charge in the form of potassium is expected to leave the cells, as indeed occurs.

The uptake of basic amino acids is accompanied by efflux of both protons and potassium (Fig. 2), so that for each arginine taken up one positive charge leaves the cells (Table IV). Lysine gave similar results. Therefore, basic amino acid transport is unipolar driven by a negative membrane potential (8, 10).

The uptake of acidic amino acids is coupled to uptake of two protons and release of one potassium per amino acid molecule (Fig. 3; Table IV). Thus, acidic amino acid transport is a proton-cotransport system charge compensated by potassium. The second proton neutralizes the dissociated distal carboxyl group. Based on electrophysiological data, it has been proposed that acidic amino acids involved H⁺ amino acid cotransport but with a second cation to compensate amino acid charge (8). Our data show that the second cation is, in fact, another proton.

It is of interest which form of glutamic acid is bound. Either the glutamic acid anion or two protons are bound separately, or the glutamic acid anion picks up one proton from the medium to become the uncharged glutamic acid (the pK value of the γ-carboxyl group is 4.25) which is then bound together with one proton. The more or less constant Km (Fig. 4) is strong evidence for the glutamate anion with the ionized γ-carboxyl group as the real substrate of the transport system.

DISCUSSION

Sugarcane suspension cells appear to have three amino acid uptake systems, the specificity is determined by the net charge of the amino acid at physiological pH as was previously suggested (8). Histidine exists in two ionization states, but our data supports its transport only in the neutral form and by the system for neutral amino acids. Despite the clear amino acid specificity of the transport systems, there is a striking homology in all systems at the site of substrate binding: in all cases, the data support a positively charged complex at the binding site, either because the amino acid itself is positively charged (arginine) or a proton is bound in addition to the neutral amino acid (alanine) or two protons are bound to an anion (glutamate). It is tempting to speculate that the structure at the binding site is, therefore, not drastically different. Since the binding site of the proton is known in the case of arginine, i.e. at the guanidino group (10), it might be suggested to be also very near the amino acid molecule in case of the neutral amino acids. For sugar transport in Chlorella, evidence for very close binding of sugar and proton has been found (9). It can be easily imagined that a transport site could lose part of its specificity toward the neutral side chain of an amino acid and become a general transport system for either...
neutral, basic, or acidic amino acids. However, charge plays a more crucial role in determining binding as was shown previously for arginine uptake (10).

The stoichiometry of proton uptake per amino acid in sugarcane is very similar to the reported values for bacteria, 1H⁺/alanine and 2H⁺/glutamate (2, 13), whereas for yeast the values

Table IV. Stoichiometries of Proton and Potassium Flux during Amino Acid Uptake

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>System I</th>
<th>System II</th>
<th>System III</th>
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</thead>
<tbody>
<tr>
<td>H⁺ Influx/</td>
<td>0.71 ± 0.40</td>
<td>0.27 ± 0.15</td>
<td>1.89 ± 0.56</td>
</tr>
<tr>
<td>K⁺ Efflux/</td>
<td>0.98 ± 0.36</td>
<td>0.73 ± 0.18</td>
<td>1.06 ± 0.11</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>0.91 ± 0.09</td>
<td>1.10</td>
<td>2.09 ± 0.87</td>
</tr>
<tr>
<td>Pro</td>
<td>0.98</td>
<td></td>
<td>0.97 ± 0.56</td>
</tr>
<tr>
<td>Ala</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln</td>
<td>0.29 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>His (pH 5.8)</td>
<td>-0.61*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Asp</td>
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</table>

*Estimated proton release from the imidazol-nitrogen at pH 5.8.

Fig. 1. Change in H⁺ and K⁺ concentrations in the medium during uptake of neutral amino acids. Vertical bars depict deflection on addition of 10 nmol HCl or 100 nmol KCl.

Fig. 2. Change in H⁺ and K⁺ concentrations in the medium during uptake of basic amino acids. Vertical bars depict deflection on addition of 10 nmol HCl or 100 nmol KCl.
AMINO ACID TRANSPORT

Fig. 4. $K_m$ value of glutamate uptake at different pH values. The dotted line shows the expected change of $K_m$ value if glutamate with the undissociated $\gamma$-carboxyl group would be taken up.

are higher, namely $1H^*/\text{lysine}$ and $2H^*/\text{glycine}$, and nearly $3H^*/\text{glutamate}$ (1, 3, 18). The very close stoichiometry between positive charge movement during amino acid influx and $K^+$ efflux eliminate a possible role for a permeant anion, such as $\text{Cl}^-$, in charge compensation.

Bacteria (15), fungi (3), and algae (Cho et al., in preparation) have a multitude of uptake systems for amino acids, more than a dozen in some cases. It can be imagined that free living organisms need a number of finely tuned transport systems which are exactly adaptable to a changing environment. Plant parenchyma cells have perhaps a much more uniform nutrient supply and general transport systems are sufficient. The uptake of amino acids by only three systems can be expected to have consequences for the deprivation of nutrients from the culture medium since, for instance, the presence of 16 neutral amino acids in the culture medium will create an enormous competitive situation (20). It will be the aim of future research to model the distinct behavior of nutrient uptake by the kinetic features of the transport systems.

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LITERATURE CITED

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