The H+-dependent AAP5 amino acid transporter from Arabidopsis thaliana was expressed in Xenopus oocytes, and we used radiotracer flux and electrophysiology methods to investigate its substrate specificity and stoichiometry. Inward currents of up to 9 μA were induced by a broad spectrum of amino acids, including anionic, cationic, and neutral amino acids. The apparent affinity of AAP5 for amino acids was influenced by the position of side chain branches, bulky ring structures, and charged groups. The maximal current was dependent on amino acid charge, but was relatively independent of amino acid structure. A detailed kinetic analysis of AAP5 using lysine, alanine, glutamate, and histidine revealed H+-dependent differences in the apparent affinity constants for each substrate. The differences were correlated to the effect of H+ concentration on the net charge of each amino acid and suggested that AAP5 transports only the neutral species of histidine and glutamate. Stoichiometry experiments, whereby the uptake of H-labeled amino acid and net inward charge were simultaneously measured in voltage-clamped oocytes, showed that the charge:amino acid stoichiometry was 2:1 for lysine and 1:1 for alanine, glutamate, and histidine. The results confirm that histidine is transported in its neutral form and show that the positive charge on lysine contributes to the magnitude of its inward current. Thus, the transport stoichiometry of AAP5 is 1 H+:1 amino acid irrespective of the net charge on the transported substrate. Structural features of amino acid molecules that are involved in substrate recognition by AAP5 are discussed.

Transport of amino acids across the plasma membrane of higher plants is mediated by proton-coupled transport proteins that utilize the electrochemical gradient for H+ to drive the uphill transport of amino acids (reviewed in Refs. 1–4). Kinetic analysis of amino acid uptake into plasma membrane vesicles isolated from sugar beet leaves suggests the presence of four H+-coupled amino acid transport systems (5–7), and at least 10 H+/amino acid transporters have been isolated by complementing yeast amino acid transport mutants with plant cDNA libraries (8–12). These transporters have very broad and overlapping specificities. However, each exhibits a preference for amino acids possessing a particular molecular geometry or charge. Analysis of the substrate specificity of amino acid transporters in yeast cells and plasma membrane vesicles is traditionally accomplished by measuring the inhibition of amino acid transport activity by various substrates. Competition experiments yield information on substrates that interact with amino acid transporters, but do not allow a distinction between substrates that are transported and those that act as inhibitors.

We previously analyzed the specificity and kinetic properties of the Arabidopsis AAP1 H+/amino acid transporter by expressing the cloned gene in Xenopus oocytes and measuring substrate-induced currents using electrophysiology methods (13). AAP1 transported anionic and neutral amino acids. However, except for histidine, the transport of cationic amino acids was negligible. In this study, we chose to investigate the specificity of the Arabidopsis AAP5 H+/amino acid transporter, which shares 54% identity and 73% similarity with AAP1. Unlike AAP1, expression of AAP5 in yeast suggests that it efficiently transports anionic, neutral, and cationic amino acids (11), making it an ideal candidate to investigate the effects of a broad spectrum of amino acids on substrate recognition. We expressed AAP5 in Xenopus oocytes and used the two-electrode voltage-clamp method to determine the apparent kinetic parameters (maximal current (i_{\text{max}}) and apparent affinity (K_{a,i})) of various amino acids. For AAP5 and AAP1, a combination of electrophysiology and radiotracer flux methods enabled us to determine the H+/amino acid stoichiometry of neutral, cationic, and anionic amino acids, which revealed the net charge on the transported species. We show that 1) amino acid geometry and charge dramatically affect the substrate specificity of AAP5, and 2) AAP5 transports neutral, anionic, and cationic amino acids with a fixed H+/amino acid stoichiometry. Thus, the kinetic approaches used in this study enabled us to gain insights into the nature of the substrate-binding site and transport mechanism of AAP5.

EXPERIMENTAL PROCEDURES

Molecular Biology—AAP5 and AAP1 were polyadenylated as described previously (13). The resulting plasmids, pKAAP5 and pKAAP1, were linearized with KpnI, and capped cRNA was transcribed in vitro using T7 RNA polymerase and an RNA transcription kit (Ambion Inc., Austin, TX).

Oocyte Preparation—Xenopus oocytes were isolated and injected with 25–50 ng (1 μg/μl) of cRNA encoding AAP5 or AAP1 or with 50 nl of water (control oocytes) and were maintained in Barth’s medium for up to 5 days post-injection as described previously (13).

Uptake Experiments—The amount of amino acid transported into oocytes under non-voltage-clamp conditions was determined using a radiotracer method. Groups of 8–10 cRNA- or water-injected oocytes were incubated in transport buffer (100 mM choline chloride, 2 mM KCl, 50 mM NaCl, 2 mM MgCl2, 10 mM Hepes-KOH, pH 7.5) for 10 min at 20 °C.
1 mM CaCl₂, 1 mM MgCl₂, 10 mM PIPES, and 10 mM HOMOPIPES) containing 0.032 or 10 μM H⁺ and 100 μM 3H-labeled alanine, lysine, histidine, or glutamate (Amersham International, Buckinghamshire, United Kingdom). After 30 min at 22 °C, the oocytes were washed three times in 5 ml of ice-cold buffer and lysed in 10% sodium dodecyl sulfate, and the amount of radioactivity was determined by liquid scintillation counting.

Electrophysiology Experiments—All experiments were done using the two-electrode voltage-clamp method (13–15). The membrane potential was clamped at −50 mV, and steady-state currents were recorded 50 ms after the onset of voltage pulses ranging from −150 to 50 mV (20 mV increments). Steady-state amino acid-induced currents were obtained by subtracting the difference between steady-state currents in the presence and absence of amino acid. The apparent affinities for amino acids and protons (K_{app} and K_{app}'), respectively, and their maximal currents (i_{max} and i_{max}') respectively) were obtained by fitting the steady-state amino acid-induced currents at each test potential (V_m) to Equation 1,

\[ i = \frac{i_{max}}{K_S + [S]^n} + (K_H^n)^n \]  

where [S] is the [amino acid], or [H⁺], i_{max} is the maximal current for saturating S, K_{app} is the apparent affinity of the substrate (S giving half the (i_{max})), and n is the Hill coefficient. All fitting procedures were done using Sigma Plot software (Jandel Scientific, San Rafael, CA).

Determination of H⁺:Amino Acid Stoichiometry—To determine the H⁺:amino acid transport ratio, oocytes were voltage-clamped, and inward fluxes of H⁺-labeled amino acids and net inward amino acid-induced currents were measured simultaneously (151). Throughout the experiment, substrate-induced currents were recorded using Fetchex software (Axon Instruments, Inc., Foster City, CA). Oocytes were clamped at potentials ranging from −10 to −90 mV and superfused with transport buffer at a rate of 160 μl/min. Current traces were monitored until they reached a steady base line, after which 0.5 mM H⁺-labeled amino acid was superfused for 30 s to 10 min while recording the amino acid-induced current. The oocyte was washed in the absence of amino acid until the current returned to base-line levels. The oocyte was quickly removed from the chamber, washed three times in 5 ml of ice-cold buffer, and lysed in 10% sodium dodecyl sulfate, and the amount of radioactivity was determined by liquid scintillation counting. The total inward charge was calculated by subtracting the base-line current and integrating the area under the current versus time curve. The H⁺:amino acid transport ratio is presented as pmol of net inward charge/pmol of amino acid transport.

The results are representative of experiments that were repeated at least three times with oocytes from different donor frogs. All experiments were carried out at 22 °C, and all amino acids used in this study were l-isomers. Chemicals were purchased from Sigma.

Crystal Structure Comparisons—The Cambridge Crystallographic Data Base was searched to obtain the x-ray crystal structures of the α-amino acids lysine, ornithine, histidine, cysteine, arginine, methionine, serine, threonine, glycine, leucine, glutamine, glutamate, alanine, citrulline, isoleucine, valine, proline, phenylalanine, aspartate, tryptophan, and asparagine. The crystal structure of homoglutamine is not available, and this molecule was drawn using the molecular modeling program Hyperchem (Version 4.5, Hypercube, Waterloo, Ontario, Canada).

RESULTS

Specificity of AAP5 for Amino Acids—The apparent kinetic parameters for various amino acids were obtained by expressing AAP5 in Xenopus oocytes and measuring the steady-state amino acid-induced currents as a function of membrane voltage and external [amino acid], at 10 μM H⁺. The amino acid-induced currents obtained at −150 mV were plotted against [amino acid] and the concentration/current curves were fitted to Equation 1. Table I shows the apparent affinity (K_{app}) and three-dimensional x-ray crystal structures of the amino acids. The K_{app} values for glutamate and histidine were adjusted to account for the net charge on the transported species: the neutral species of histidine and glutamate are transported by AAP5 (see below and “Discussion”). AAP5 had the highest apparent affinity for arginine, histidine, homoarginine, and methionine (−0.1–0.3 mM), followed by lysine, ornithine, alanine, and glycine (−0.4–0.5 mM). Serine, glutamine, glutamate, cysteine and arginine all had K_{app} values <1 mM. The K_{app} values for threonine, homoserine (1.6 ± 0.1 mM), and leucine were higher, between −2 and 4 mM. For proline, hydroxyproline (32 ± 10 mM), tryptophan, and valine, the K_{app} values increased by at least 2 orders of magnitude over that of arginine to between 20 and 35 mM. The current/concentration curves for isoleucine, phenylalanine, and asparagine were far from saturation even at 50 mM amino acid, with estimated K_{app} values of <40, 80, and >100 mM, respectively. Neither aspartate nor the β-amino acids γ-aminobutyric acid and β-alanine were transported by AAP5. Aspartate does not interact with AAP5: the magnitude of the lysine-induced currents was the same in the absence and presence of 50 mM aspartate.

Fig. 1 shows a comparison of the maximal currents induced by amino acids as a percentage of the lysine-induced current. The highest i_{max} values were obtained for lysine and ornithine, followed by histidine, arginine, and homoarginine. Except for tryptophan, which had an i_{max} of ~8% of the lysine-induced current, all other amino acids had i_{max} values between 30 and 50% of that for lysine. The activation curves for phenylalanine, isoleucine, and asparagine did not saturate. Thus, the i_{max} values for these amino acids are not included in Fig. 1.

Fig. 2 shows representative, normalized current/voltage re-

\[ K_{app} = \frac{[S]}{i_{max}} + (K_H^n)^n \]
the current recorded at 
absence of amino acid. The current/voltage curves were normalized to 
difference between the currents measured at 50 ms in the presence and 
duced currents in oocytes expressing AAP5.

Steady-state amino 
lationships obtained with 20 mM alanine, glutamate, histidine, 
lysine, histidine, glutamate, and alanine at 10
m
acid-dependent current/voltage relationships were obtained with 20 mM 
amino acid. The curves were hyperbolic, suggesting that 1 amino acid 
binds to the transporter per transport cycle. Symbols represent the data 
points. Data for alanine were removed for clarity. B, H+ activation 
curves obtained with 20 mM amino acid, at −150 mV and normalized to 
the current recorded at 10 μM H+. With lysine, histidine, and alanine 
as substrates, the H+ activation curves were hyperbolic, suggesting 
that 1 H+ binds to AAP5 per transport cycle. However, for glutamate, 
the curves were sigmoidal with an apparent coupling coefficient >1. 
Symbols represent the data points. Data points for alanine and histi-
dine were removed for clarity.

Substrate Specificity and Stoichiometry of AAP5

Fig. 1. Maximal currents induced by amino acids. The \( i_{\text{max}} \) for 
each amino acid was obtained as described in the legend to Table I and 
is expressed as a percentage of \( i_{\text{max}} \pm \text{error of the fit.} \) The currents 
induced by lysine varied from 2 to 9 nA depending on the batch of 
oocytes. Substrate-induced currents from water-injected oocytes were 
typically <5 nA (data not shown). H-Arg, homoarginine.

Fig. 2. Voltage dependence of steady-state amino acid-induced 
currents in oocytes expressing AAP5. Steady-state amino 
acid-dependent current/voltage relationships were obtained with 20 mM 
lysine, histidine, glutamate, and alanine at 10 μM H+, and are the 
difference between the currents measured at 50 ms in the presence and 
absence of amino acid. The current/voltage curves were normalized to 
the current recorded at −150 mV.

Fig. 3. Amino acid and H+ activation curves for AAP5. Steady-
state amino acid-induced currents were obtained at different [amino 
acid]$_o$ and [H+]$_o$. At every membrane potential, the current/concentration 
curves were fitted to Equation 1 to obtain the maximal current 
(\( i_{\text{max}} \)), apparent affinity (\( K_a \)), and apparent coupling coefficient (\( n \)) for 
amino acid and H+. Amino acid activation curves obtained with 10 μM 
H+ at −150 mV and normalized to the current recorded at 20 mM 
amino acid. The curves were hyperbolic, suggesting that 1 amino acid 
binds to the transporter per transport cycle. Symbols represent the data 
points. Data for alanine were removed for clarity. B, H+ activation 
curves obtained with 20 mM amino acid, at −150 mV and normalized to 
the current recorded at 10 μM H+. With lysine, histidine, and alanine 
as substrates, the H+ activation curves were hyperbolic, suggesting 
that 1 H+ binds to AAP5 per transport cycle. However, for glutamate, 
the curves were sigmoidal with an apparent coupling coefficient >1. 
Symbols represent the data points. Data points for alanine and histi-
dine were removed for clarity.

Kinetics of Alanine, Lysine, Glutamate, and Histidine Transport—Alanine, lysine, glutamate, and histidine carry different 
net charges in solution over the range of [H+]$_o$ (0.032–10 μM) 
used in this study (see Fig. 7 and “Discussion”). Therefore, as a 
first step to investigate the effect of substrate charge on the 
specificity of AAP5, the apparent kinetic parameters for each 
amino acid were obtained by varying [amino acid]$_o$ at fixed 
[H+]$_o$, varying [H+]$_o$ at fixed [amino acid]$_o$, and fitting the 
concentration/current data to Equation 1.

Fig. 3A shows the normalized amino acid activation curves 
obtained at −150 mV and 10 μM H+. At test potentials be-
tween −150 and −30 mV and when [H+]$_o$ was decreased, the 
curves were hyperbolic with \( n = 1 \). Likewise, the H+ activation 
curves obtained with alanine, lysine, and histidine were hyper-
bolic with \( n = 1 \) irrespective of the applied potential and [amino 
acid]$_o$. However, the H+ activation curves obtained with glutamate 
were sigmoidal. Fig. 3B shows representative H+ activation 
curves obtained with 20 mM amino acids at −150 mV and 
with 5 mM glutamate at −30 mV.

Table II shows that \( K_{H}^{\text{glu}} \) values determined in 20 mM alanine 
and lysine were lower than in glutamate and histidine, and when [amino acid]$_o$ decreased, \( K_{H}^{\text{glu}} \) increased. Thus, AAP5 had

| Amino Acid | Lys/Ala | Glu | His |
|-----------|---------|-----|-----|
|           |         |     |     |

| Amino Acid | Lys/Ala | Glu | His |
|-----------|---------|-----|-----|
|           |         |     |     |
a very high apparent affinity for $H^+$ (0.2 mM, pH 6.7) when [lysine]o, and [alanine], were saturating such that both amino acids induced inward currents in the absence of a downhill $H^+$ gradient across the oocyte plasma membrane (cytoplasmic [H+]i = -0.04 mM) (16). Due to substrate-dependent differences in the apparent affinity of AAP5 for $H^+$, apparent kinetic parameters were obtained at 0.032 and 10 mM $H^+$o for lysine and alanine and at 1 and 10 mM $H^+$o for glutamate and histidine. Mean kinetic values obtained from three experiments at -150 mV are shown in Fig. 4. Fig. 4A shows that increasing [H+]i increased the maximal current for amino acids. A 300-fold increase in [H+]i, increased $i_{\text{max}}^{\text{max}}$, 4-fold and $i_{\text{max}}^{\text{max}}$, 5-fold; a 10-fold increase in [H+]i, increased $i_{\text{max}}^{\text{max}}$, 2-fold and $i_{\text{max}}^{\text{max}}$, 1.5-fold. For each amino acid, the $i_{\text{max}}$ versus voltage curves were supralinear (data not shown). Fig. 4B shows that the apparent affinity constants decreased as [H+]i increased. A 300-fold increase in [H+]i, decreased $K_{0.5}^{K\text{H}}$ from 1.5 to 0.4 mM and $K_{0.5}^{K\text{H}}$ from 6 to 0.5 mM. A 10-fold increase in [H+]i, decreased $K_{0.5}^{K\text{H}}$ from 50 to 5 mM, whereas the decrease in $K_{0.5}^{K\text{H}}$, was much less (3 to 2 mM). The $K_{0.5}$ values for histidine and glutamate shown in Table I were adjusted to account for the net charge on the transported species (see “Discussion”).

The steady-state kinetic analysis of AAP5 can be summarized as follows. 1) The transport of neutral, anionic, and cationic amino acids is electrogenic. 2) The voltage dependence of the saturating substrate-induced currents is the same, suggesting that a voltage-dependent step in the reaction cycle is independent of the net charge on the substrate. According to the models proposed for the $H^+$/dipeptide transporter and the Na+/glucose cotransporters, this step is probably the reorientation of the empty carrier from the cytoplasmic to the extracellular surface (14, 17). 3) $H^+$ and amino acids increased the $i_{\text{max}}$ and $K_{0.5}$ values for their respective cosubstrates. Positive cooperativity between the two ligands suggests that AAP5 operates via a simultaneous mechanism (18). Also, $H^+$ acts as an essential activator probably by orientating the amino acid-binding site to the external membrane surface (13, 15, 19).

Uptake Experiments—To show that the inward currents induced by amino acids were due to their uptake into oocytes, radiotracer flux experiments were performed on unclamped oocytes with 100 mM $H^+$-labeled alanine, lysine, histidine, and glutamate at 0.032 and 10 mM $H^+$. After 30 min at 0.032 mM $H^+$o, uptake was as follows: alanine $\gg$ lysine $\gg$ glutamate $\gg$ histidine (108 $\pm$ 5, 94 $\pm$ 14, 46 $\pm$ 5, and 1.4 $\pm$ 0.3 pmol/oocyte, respectively). Thus, although glutamate-induced currents could not be measured at 0.032 mM $H^+$o, tracer experiments showed that glutamate is transported by AAP5 at low [H+]o. At 10 mM $H^+$o, uptake was as follows: alanine $\gg$ lysine $\gg$ glutamate $\gg$ histidine (520 $\pm$ 47, 316 $\pm$ 29, 127 $\pm$ 5, and 76 $\pm$ 6 pmol/oocyte, respectively). These results were surprising since the currents induced by lysine were significantly higher than the currents induced by alanine, and both substrates had similar $K_{0.5}$ values and voltage dependences at 10 mM $H^+$o. This suggests that an extra charge accompanies lysine transport and contributes to the magnitude of the lysine-induced current, which has important implications for the transport mechanism of AAP5.

Stoichiometry Experiments—The steady-state kinetic data suggested that the $H^+$:amino acid stoichiometry was 1:1 for alanine, lysine, and histidine, but >1:1 for glutamate. To determine the $H^+$:amino acid stoichiometry directly rather than relying on Hill coefficients and to investigate the discrepancy between the magnitude of the steady-state currents and amount of substrate transported by AAP5, we simultaneously measured the amino acid-induced current and uptake of $H^+$-labeled amino acid in voltage-clamped oocytes. This method was chosen because it gives a more direct measurement of stoichiometry, whereas a thermodynamic approach requires a knowledge of the internal ligand concentrations. Fig. 5A is a typical current trace obtained from an oocyte voltage-clamped at -50 mV and superfused with 0.5 mM $[^{3}H]$lysine at 10 mM $H^+$o. Addition of lysine to the oocyte transport buffer induced a large inward current (~600 nA) that declined with time. When lysine was removed, the currents returned to the base-line levels. The current traces were qualitatively similar irrespective of the amino acid under investigation. Under voltage-clamp conditions, amino acids accumulated above [amino acid]o. For example, oocytes clamped at -50 mV and superfused with 0.15 mM lysine, for 10 min accumulated lysine up to -15-fold (2.2 mA) above [lysine]o. This was calculated assuming that the volume of the stage V oocytes used in this study was ~900 nl. The uptake of amino acids into voltage-clamped

---

**Table II**

**Apparent affinity of AAP5 for $H^+$**

| Substrate | Apparent affinity (mM) |
|-----------|------------------------|
| Ala       | 0.2 ± 0.05             |
| Glutamate | 3.9 ± 0.3              |
| Histidine | 0.44 ± 0.07            |
| Lysine    | 0.2 ± 0.05             |

$^a$ — data not obtained.

---
Substrate Specificity and Stoichiometry of AAP5

Fig. 5. Determination of charge:amino acid stoichiometry. Oocytes were voltage-clamped at potentials between −10 and −90 mV and superfused with 0.5 mM 3H-labeled amino acid. A, typical current trace obtained at −50 mV with 0.5 mM lysine and 10 μM H⁺. The dotted line represents the base-line current at 10 μM H⁺. The base-line current was subtracted from the total current trace, and the current versus time curve was integrated to yield the total amount of charge transported per oocyte. B, for 10−12 oocytes, the amount of amino acid transported was plotted against the amount of charge. Regression analysis of curves obtained with 0.5 mM lysine and alanine at 10 μM H⁺ yielded curves with slopes of 2.1 ± 0.04 and 1.1 ± 0.07, respectively. When lysine was removed from the transport buffer, there was no outward current, suggesting that there was no electrogenic efflux of lysine from the oocyte.

Water-injected oocytes was negligible (<5 pmol, 5.0 μM) over the same time scale.

Fig. 5B shows plots of amino acid transported (pmol/oocyte) versus amount of charge transported (pmol/oocyte) for oocytes superfused with 0.5 mM lysine or alanine and 10 μM H⁺, at −50 mV. Regression analysis yielded straight lines with slopes corresponding to the charge:amino acid stoichiometry: 2.1 ± 0.04:1 (r² = 0.99) for lysine and 1.1 ± 0.07:1 (r² = 0.97) for alanine. Thus, the net single positive charges on lysine and, presumably, ornithine, homoarginine, and arginine contribute to the magnitude of their induced currents.

At 10 μM H⁺ and 0.5 mM lysine, the charge:lysine stoichiometry was voltage-dependent and increased from 1.9 ± 0.1:1 at −10 mV to 2.5 ± 0.05:1 at −90 mV. At 0.32 μM H⁺ and 0.5 mM lysine, the charge:amino acid stoichiometry was 1.6 ± 0.03:1 at −90 mV. These results suggest that there is an uncoupled transport of protons through AAP5 and is supported by the increase in the base-line current when [H⁺]₀ was increased from 0.032 to 10 μM (see Fig. 5A).

Fig. 6 shows a comparison of the charge:amino acid stoichiometry of AAP5 and AAP1 obtained with 0.5 mM 3H-labeled amino acids and 10 μM H⁺, at −50 mV. For both transporters, the transport stoichiometry of histidine, glutamate, and alanine was ~1 charge:1 amino acid compared with ~2 charges:1 lysine for AAP5 (AAP1 does not transport lysine) and are conclusive evidence that histidine is transported in its neutral form (see “Discussion”). Assuming that the single inward charge accompanying the transport of each amino acid is due to the transport of H⁺, we conclude that the H⁺:amino acid stoichiometry (1:1) is the same irrespective of the net charge of the amino acid in solution.

DISCUSSION

Expression of the H⁺/amino acid transporter AAP5 in Xenopus oocytes enabled us to use electrophysiology and radiotracers for measurement to determine 1) the preferred molecular weight of amino acids for transport, 2) the net charge on the transported amino acid species, 3) the H⁺:amino acid coupling stoichiometry.

Role of Charged Groups in Substrate Specificity and Stoichiometry of AAP5—To undertake a thorough investigation of the substrate specificity of AAP5, it was necessary to determine the net charge on the transported amino acid species. For each amino acid investigated under the experimental conditions used in this study (0.032–10 μM H⁺, the α-carboxyl and α-amino groups were ionized, and their net charge was depend-
ent on the nature of the side chain. Fig. 7 shows the effect of $[$H$^-]_o$ on the distribution of charged species of alanine, lysine, glutamate, and histidine. About 100% of lysine carries a single net positive charge (cationic), and -100% of alanine carries no net charge (neutral). Glutamate is predominantly negatively charged (anionic). However, there is a significant amount of neutral glutamate (~0.06% at 0.032 mM H$^+$, increasing to 15% at 10 mM H$^+$.). Between 0.032 and 10 mM H$^+$, the amount of cationic histidine increases from 9 to 91%, and the amount of neutral histidine decreases from 91 to 9%. Of the other amino acids tested, arginine, ornithine, and homoarginine carry a net positive charge; the charge on aspartate is similar to that on glutamate; and the other amino acids are neutral.

The stoichiometry data showed that, for both AAP5 and AAP1, a single net inward positive charge accompanied the uptake of alanine, histidine, and glutamate, whereas two inward charges were cotransported with lysine by AAP5. At 10 mM H$^+$, the net charge on alanine is zero, and the net charge on lysine is +1. Assuming the extra inward charge was carried by H$^+$, the H$^+$-amino acid stoichiometry for alanine and lysine was 1:1 with the charge on lysine contributing to the magnitude of its induced current. Thus, AAP5 recognizes the cationic species of lysine and probably the cationic species of arginine, homoarginine, and ornithine.

Since the neutral species of histidine is transported by AAP5, the values of $K_{\text{H}_{1/2}o}$ obtained at 1 and 10 mM H$^+$ (3 and 2 mM, respectively) were recalculated. The amount of neutral histidine at 1 mM H$^+$ is 50% compared with 9% at 10 mM H$^+$. Therefore, “real” $K_{\text{H}_{1/2}o}$ values of 1.5 and 0.18 mM, respectively. Also, the H$^+$ activation data for histidine shown in Table II must be re-examined. These data were obtained by varying [H$^+$]$_o$ at fixed [histidine]. However, to obtain H$^+$ activation data using the same concentration of neutral histidine (e.g. 20 mM), we would need to vary [H$^+$]$_o$ and [histidine].

The stoichiometry data suggest either that neutral glutamate is transported with one proton or that anionic glutamate is transported with two protons. The glutamate activation curves obtained at fixed [glutamate] were hyperbolic, whereas the H$^+$ activation curves obtained at fixed [glutamate] were sigmoidal, suggesting that the H$^+$-amino acid stoichiometry was >1:1. However, when [H$^+$]$_o$ was decreased from 10 to 1 mM, $K_{\text{H}_{1/2}o}$ increased 10-fold, which is consistent with a decrease in the amount of neutral glutamate; $K_{\text{H}_{1/2}o}$ increased only 1.5-fold over the same range of [H$^+$]. Thus, the shape of the H$^+$ activation curves can also be explained if glutamate is transported in its neutral form. For example, a 20 mM glutamate solution would contain 12 mM neutral glutamate at 0.032 mM H$^+$ and 3 mM at 10 mM H$^+$ (see Fig. 7), which would explain the observed lag in the H$^+$ activation curves. If glutamate is transported as the neutral species, the real $K_{\text{H}_{1/2}o}$ at 10 mM H$^+$ is 0.75 mM, which is similar to the $K_{\text{H}_{1/2}o}$ for glutamine (0.78 mM), a neutral amino acid that has a similar three-dimensional structure to glutamate (see Table I). Like histidine, the H$^+$ activation data for glutamate shown in Table II must be re-examined using the same concentrations of neutral glutamate at each [H$^+$].

The stoichiometry experiments showed that, irrespective of the net charge on the transported substrate, amino acid transport by AAP5 occurs with a H$^+$:amino acid coupling stoichiometry of 1:1. Our results for histidine concur with those of Wyse and Komor (20), who concluded that neutral histidine is cotransported with 1 H$^+$ across the plant plasma membrane. However, the results for lysine contradict previous studies that suggested that the transport of lysine was facilitative (20, 21). Also, our steady-state kinetic data showed that lysine transport was H$^+$-coupled: increasing [H$^+$], decreased $K_{\text{H}_{1/2}o}$ and increased $v_{\text{max}}$; lysine transport was concentrative; and a 1 H$^+$:1 lysine coupling ratio was predicted from the hyperbolic lysine and H$^+$ activation curves (n = 1). Similarly, Sanders et al. (22) showed that H$^+$ accompanied the transport of cationic amino acids in Neurospora and that the H$^+$:amino acid stoichiometry was the same for neutral and cationic amino acids. Based on activation curves, Mackenzie et al. (23) showed that the human hPEPT1 H$^+$/dipeptide transporter cotransports anionic, cationic, and neutral dipeptides with 1 H$^+$. That glutamate is transported in its neutral form with 1 H$^+$ also contradicts the results of Kinrade and Etherton (21) and Wyse and Komor (20), who suggested that glutamate was cotransported with two cations. However, others have suggested that the neutral forms of anionic substrates are transported by the mammalian ASCT2 neutral amino acid transporter (24) and the mammalian hPEPT1 and rPEPT1 dipeptide transporters (25, 26).

Structural Determinants on Amino Acids Affecting Specificity of AAP5—As in amino acid transport into sugar beet leaves (7), the α-amino and α-carboxyl groups are essential for transport of amino acids by AAP5: γ-aminobutyric acid and β-alanine were non-interacting substrates. At 10 mM H$^+$, the apparent affinity of AAP5 for amino acids ranged over 4 orders of magnitude (~0.1 to >100 mM), and these differences were dependent upon amino acid structure and charge, but were independent of their hydrophobicity. AAP5 had a high apparent affinity for arginine, homoarginine, histidine, lysine, and ornithine, which are highly polar and, except for histidine, possess long linear side chains. Arginine and homoarginine have highly reactive, terminal guanidinium groups, which probably accounts for their high apparent affinity. Neutral, nonpolar methionine is also transported with high apparent affinity. Therefore, the substrate-binding site of AAP5 is relatively long and can accommodate amino acids >8 Å in length. Although citrulline, glutamine, and glutamate are polar molecules with long side chains, their apparent affinities were ~4-fold lower compared with arginine, probably due to a slight destabilizing effect of the distal amide or carboxylate groups: distal amide or carboxylate groups are not discriminated by AAP5. That positively charged amino acids are transported with high apparent affinity is probably a consequence of their structure rather than the presence of the positive charge: methionine and citrulline are neutral amino acids. Reducing the length of the glutamine side chain by one carbon to yield asparagine dramatically decreased the apparent affinity by at least 3 orders of magnitude. Thus, an amide group on the δ-carbon yields favorable interactions with the substrate-binding site, whereas an amide group on the γ-carbon is in an unfavorable position for binding.

Groups attached to the δ-carbon were very important in determining substrate specificity. Two methyl groups on the δ-carbon of valine decreased the apparent affinity by an order of magnitude over leucine, which has two methyl groups on the γ-carbon. Similarly, the apparent affinity for isoleucine, phenylalanine, tryptophan, and the imino acids (proline and hydroxyproline) was reduced by methyl groups, aromatic residues, or branching at the δ-carbon. Adding a methyl group to the δ-carbon of serine to give threonine decreased the apparent affinity by an order of magnitude, whereas a sulphydryl on the δ-carbon (cysteine) maintained a high apparent affinity. A hydroxyl residue on the γ-carbon was not as restrictive as a methyl group at this position: the apparent affinities for homoserine and hydroxyproline were not significantly different from those for serine and proline. AAP5 had a high apparent affinity for alanine and glycine, which are not branched at the δ-carbon. Aspartate did not interact with AAP5, suggesting that substrate binding is prevented by a γ-carboxylate. This restric-
tion was relaxed when the side chain was extended by one carbon to yield glutamate. Asparagine has a similar structure to asparagine, with a high electron density near the β-carbon. Thus, the apparent affinity for asparagine was very low (>100 mM).

The $i_{\text{max}}$ values for lysine and ornithine were high compared with those for most other amino acids, with the single net positive charge carried by these amino acids contributing to the magnitude of their induced currents. The $i_{\text{max}}$ was ~75% of the lysine-induced current. If we remove the contribution of the net single positive charge on lysine and ornithine to the magnitude of their inward currents, then AAP5 transports neutral histidine with the highest maximal transport rate. Positively charged homoarginine and arginine have considerably lower $i_{\text{max}}$ values than lysine and ornithine, probably due to the large, terminal guanidinium group, which may restrict their movement through the transporter. Except for tryptophan, the $i_{\text{max}}$ values for the other amino acids were between 28 and 51% of $i_{\text{max}}$. The bulky, aromatic side chain on tryptophan probably accounts for the low maximal rate of transport for this molecule.

Why are the charged species of glutamate and histidine excluded by AAP5? Unpaired oxygens on the deprotonated form of glutamate and protonation of the imidazole ring of histidine may produce unfavorable stereo interactions that block access to the substrate-binding site. The positive charge on the protonated imidazole ring lies ~3.6 Å from the α-carbon, whereas for arginine, homoarginine, ornithine, and lysine, the positive charge lies at least 5 Å from the α-carbon. Although the three-dimensional amino acid structures may be altered in solution due to hydration, $[\text{H}^+]$, at the substrate-binding site, and interactions with amino acid residues, the data suggest that charged groups close to the α-carboxyl and α-amino groups prevent substrate binding. Why is the neutral form of histidine transported with such high apparent affinity? Histidine, phenylalanine, and tryptophan all have bulky, aromatic rings with high electron densities close to the β-carbon, yet the $K_{0.5}$ values for phenylalanine and tryptophan are between 150- and 450-fold higher than that for histidine. Unlike phenylalanine and tryptophan, histidine possesses two nitrogen atoms in the imidazole ring, which must confer a high apparent affinity for AAP5. AAP5 has similar apparent affinities for histidine, arginine, and homoarginine, all of which possess two nitrogen atoms at their distal end.

How do the results of this study relate to the transport of amino acids in Arabidopsis? Unfortunately, the composition of free amino acids in Arabidopsis is unknown. Aspartate, glutamate, and glutamine are found at high concentrations in many plants: up to 30, 90, and 20 mM, respectively, depending on the plant species (27–29). In contrast, the concentration of lysine is ~1 mM in sugar beet leaves (30) and ~2 mM in barley leaves (28). With the exception of sink leaves, AAP5 is expressed throughout the plant, where it may play a central role in the high affinity transport of lysine. The low affinity amino acids will only be transported by AAP5 if they occur at high concentrations. Since AAP5 does not transport aspartate and since many of the low affinity amino acids such as valine, asparagine, and phenylalanine occur at low concentrations in plants, other members of the AAP family of amino acid transporters are probably responsible for the transport of these amino acids. For example, AAP1 transports valine, asparagine, and aspartate with $K_{0.5}$ values of ~0.7, 25, and 80 mM. It will be interesting to correlate the apparent affinity of each AAP transporter and their expression pattern in Arabidopsis to the abundance of particular amino acids within the plant.

Conclusions—We have shown that AAP5 recognizes and transports a broad spectrum of amino acid species differing in geometry and charge, albeit with different apparent affinities and maximal velocities. Stoichiometry experiments enabled us to determine the charge on the transported amino acid species and showed that AAP5 transports anionic, cationic, and neutral amino acids via the same mechanism, i.e. with a fixed $\text{H}^+$:amino acid coupling stoichiometry. Thus, in planta, the energy consumption for $\text{H}^+$:amino acid transport will be independent of the net charge on the amino acid. Future experiments will include a detailed investigation of the substrate specificity of other members of the AAP family of transporters and mutant transporters to identify amino acid residues involved in substrate recognition.

Acknowledgments—We thank Manoli Contreras for the preparation and injection of oocytes and Bruce Hirayama, Don Loo, Eric Turk, Wolf Frommer, and Ernest Wright for useful discussions during the preparation of this manuscript.

REFERENCES

1. Bush, D. R. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 513–542
2. Frommer, W. B., Kwart, M., Hirner, B., Fischer, W.-N., Hummel, S., and Ninnemann, O. (1994) Plant Mol. Biol. 26, 1651–1670
3. Frommer, W. B., and Ninnemann, O. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 419–444
4. Tannen, W., and Caspari, T. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 595–626
5. Li, Z.-C., and Bush, D. R. (1999) Plant Physiol. (Bethesda) 94, 268–277
6. Li, Z.-C., and Bush, D. R. (1991) Plant Physiol. (Bethesda) 96, 1338–1344
7. Li, Z.-C., and Bush, D. R. (1992) Arch. Biochem. Biophys. 294, 519–526
8. Frommer, W. B., Hummel, S., and Riesmeier, J. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5944–5948
9. Hsu, L.-C., Chiu, T.-J., Chen, L., and Bush, D. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7441–7445
10. Kwart, M., Hirner, B., Hummel, S., and Frommer, W. B. (1995) Plant J. 4, 993–1002
11. Fischer, W.-N., Kwart, M., Hummel, S., and Frommer, W. B. (1995) J. Biol. Chem. 270, 16315–16320
12. Frommer, W. B., Hummel, S., Unseld, M., and Ninnemann, O. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12036–12040
13. Boorer, K. J., Frommer, W. B., Bush, D. R., Kremen, M., Loo, D. D. F., and Wright, E. M. (1996) J. Biol. Chem. 271, 2213–2220
14. Burkhartt, B.-C. and Frommer, E. (1992) Pflugers Arch. 408, 78–83
15. Mackenzie, B., Loo, D. D. F., Fei, Y.-J., Liu, W., Ganapathy, V., Leibach, F. H., and Wright, E. M. (1996) J. Biol. Chem. 271, 5430–5437
16. Loo, D. D. F., Hazama, A., Supplisson, S., Turk, E., and Wright, E. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5767–5771
17. Jauch, P., and Leuzinger (1986) J. Membr. Biol. 94, 117–127
18. Boorer, K. J., Loo, D. D. F., and Wright, E. M. (1994) J. Biol. Chem. 269, 20417–20424
19. Boorer, K. J., Loo, D. D. F., Frommer, W. B., and Wright, E. M. (1996) J. Biol. Chem. 271, 25139–25144
20. Wyse, R. E., and Komor, E. (1984) Plant Physiol. (Bethesda) 76, 865–870
21. Kinrade, T. B., and Etherton, B. (1980) Plant Physiol. (Bethesda) 65, 1085–1089
22. Sanders, D., Slayman, C. L., and Pall, M. L. (1983) Biochim. Biophys. Acta 735, 57–76
23. Mackenzie, B., Fei, Y.-J., Ganapathy, V., and Leibach, F. H. (1996) Biochim. Biophys. Acta 1284, 125–128
24. Usunoviyi-Tate, N., Endou, H., and Kanai, Y. (1996) J. Biol. Chem. 271, 14863–14869
25. Fei, Y.-J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F. H., Romero, M. F., Singh, S. K., Boron, W. F., and Hediger, M. A. (1994) Nature 368, 563–566
26. Wenzel, U., Geber, T., Weintraut, H., Weber, W.-M., Claus, W., and Daniel, H. (1996) J. Pharmacol. Exp. Ther. 277, 831–839
27. Rienz, B., Lohaus, G., Heineke, D., and Heldt, H. W. (1991) Plant Physiol. (Bethesda) 97, 227–233
28. Winter, H., Lohaus, G., and Heldt, H. W. (1992) Plant Physiol. (Bethesda) 99, 996–1004
29. Leidreiter, K., Kruse, A., Heineke, D., Robinson, D. G., and Heldt, H. W. (1995) Bot. Acta 108, 439–444
30. Lohaus, G., Burba, M., and Heldt, H. W. (1994) J. Exp. Bot. 277, 1097–1101
31. K. J. Boorer, unpublished observations.