The amino acid transporter AAP1/NAT2 recently cloned from Arabidopsis thaliana was expressed in Xenopus oocytes and we used electrophysiological, radio-tracer flux, and electron microscopic methods to characterize the biophysical properties, kinetics, and specificity of the transporter. Uptake of alanine was $H^+$-dependent increasing from 14 pmol/oocyte/h at 0.032 $\mu$M $H^+$ to 370 pmol/oocyte/h at 10 $\mu$M $H^+$. AAP1 was electrogenic; there were an amino acid-induced depolarization of the oocyte plasma membrane and net inward currents through the transporter due to the transport of amino acids. AAP1 transported a wide spectrum of amino acids favoring neutral amino acids with short side chains. The maximal current ($I_{\text{max}}$) for alanine, proline, glutamine, histidine, and glutamate was voltage- and [H$^+$]-dependent. Similarly, the $I_{\text{max}}$ was voltage- and [amino acid]-dependent. The $I_{\text{max}}$ for both $H^+$ and amino acid were dependent on the concentrations of their respective cosubstrates, suggesting that both ligands bind randomly to the transporter. The $K_{0.5}$ of the transporter for amino acids decreased as [H$^+$] increased and was lower at negative membrane potentials. The $K_{0.5}$ for $H^+$ was relatively voltage-independent and decreased as [amino acid] increased. This positive cooperativity suggests that the transporter operates via a simultaneous mechanism. The Hill coefficients $n$ for amino acids and $H^+$ were $>1$, suggesting that the transporter has more than one binding site for both $H^+$ and amino acid. Freeze-fracture electron microscopy was used to estimate the number of transporters expressed in the plasma membrane of oocytes. The density of particles on the protoplasmic face of the plasma membrane of oocytes expressing AAP1 increased 5-fold above water-injected controls and corresponded to a turnover number 350 to 800 s$^{-1}$.

In plants, amino acid transport is essential for the redistribution of nitrogen. Amino acids are transported within the phloem and xylem from their site of synthesis (roots and leaves) to organs that are net importers of nitrogen such as leaves, seeds, and tubers. Likewise, amino acids resulting from the degradation of storage proteins during germination are exported via the vascular system to supply the developing plant. Studies using whole tissues, individual cells, protoplasts, and isolated plasma membrane vesicles showed that amino acid transport in plants is mediated by specific membrane transport proteins that couple the electrochemical potential gradient of $H^+$ to secondary active accumulation of amino acids (Reinhold and Kaplan, 1984; Bush and Langston-Unkefer, 1988; Li and Bush, 1990, 1991, 1992; Williams et al., 1990, 1992; Weston et al., 1994; Bush, 1993). In recent years, molecular biological techniques, whereby yeast amino acid transport mutants are complemented with higher plant cDNA libraries, have enabled the genes encoding amino acid transport proteins to be isolated (Frommer et al., 1993; Hsu et al., 1993; Hsu et al., 1993; Kwart et al., 1993; Fischer et al., 1995). One amino acid transporter clone (AAP1) was isolated from Arabidopsis thaliana by complementing a yeast mutant defective in proline uptake and was characterized as a general amino acid transporter (Frommer et al., 1993). Another clone, NAT2, was isolated from Arabidopsis by Hsu et al. (1993), which has $>$99% identity to AAP1. Both have a predicted molecular mass of 53 kDa, are highly hydrophobic, and contain 10–12 putative membrane-spanning regions. When expressed in yeast both transporters exhibited saturable, concentration-dependent amino acid transport and had a broad substrate specificity.

Experimental Procedures

Molecular Biology Methods—The 3′-untranslated region of NAT2 lacks a poly(A) tail, and that of AAP1 has a poly(A) tail of only 16 nucleotides. We have found that a poly(A) tail of at least 30 nucleotides is necessary for the efficient expression of plant transport proteins in vivo. The expression of AAP1 and NAT2 in Xenopus oocytes and used electrophysiological, radio-tracer flux, and morphometric methods to characterize their kinetics and substrate specificity. We show that AAP1 has a broad substrate specificity and is electrogenic with membrane voltage influencing the apparent $K_{0.5}$ for protons and amino acids and the maximal current generated by the transporter. Furthermore, a freeze-fracture analysis of AAP1 expressed in the oocyte plasma membrane allowed us to determine the turnover number of the transporter.

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From the Department of Physiology, UCLA School of Medicine, Los Angeles, California 90095-1751, and the Institute for Genobiologische Forschung, Ihnestrasse 63, 14195 Berlin, Germany, and the United States Department of Agriculture, Agricultural Research Service and Department of Plant Biology, University of Illinois, 196 PABL, Urbana, Illinois 61801

2 AAP1 and NAT2 behave identically with respect to their transport kinetics and substrate specificity. Therefore, for simplicity and in view of the large size of the AAP gene family, they will be referred to as AAP1 throughout the text.
oocytes. Therefore, a pol(A) tail of 70 adenoses was introduced to the 3'-untranslated region of NAT2 and AAP1 as follows. A pol(A) tail of 70 adenoses was inserted between the HindIII and Xhol sites of pBluescript KS+ (Strategene) to yield pk' B1. The EcoRI fragments of NAT2 and AAP1 were inserted into the EcoRI site of pk' B1, and the length of the pol(A) tail was verified by sequencing the 3'-end of the construct using Sequenase version 2.0 kit (U. S. Biochemical Corp.). The resulting plasmids pKNAT2 and pKKAAP1 were linearized with KpnI, and capped RNA was transcribed in vitro using T7 RNA polymerase and a RNA transcription kit (Ambion, Austin, TX).

Preparation of Oocytes—Stage V or VI oocytes from Xenopus laevis were incubated in 5 mg/ml collagenase-B (Boehringer Mannheim) for 1 h at 22°C, and used defolliculated by incubation in 100 mM K$_2$HPO$_4$, 0.1 g/ml bovine serum albumin for 1 h with gentle agitation. Oocytes were maintained at 18°C in Barth’s medium containing (in mM) 88 NaCl, 1 KCl, 0.33 Ca(NO$_3$)$_2$, 0.41 CaCl$_2$, 0.82 MgSO$_4$, 2.4 NaHCO$_3$, 10 HEPES, 0.1 mg/ml gentamycin, pH 7.4, before and after injection of 50 ng (1 μg/ml) of RNA.

Solutions—Xenopus oocytes possess endogenous amino acid transporters, which function either as facilitators or are driven by the electrochemical gradient for Na$^+$ (Campbell and Kilberg, 1989; Taylor et al., 1989). Although these activities are low, experiments were done in the absence of Na$^+$ to avoid background activity due to endogenous Na$^+$-dependent amino acid transporters. Radiotracer uptake experiments and physiological recordings were done in transport buffer containing (in mM) 100 choline chloride, 2 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 PIPES, and 10 HOMOPIPES. The [H$^+$]$_0$ of the bathing medium was varied between 0.032 and 15.8 μM (pH 7.5 and 4.8) using Tris base. In some experiments, NaCl was substituted for choline chloride, and extra K$^+$ was added as KCl or replaced by 2 mM choline chloride.

Radiotracer Flux Analysis—Experiments were carried out 6–7 days after injection of 50 ng (1 ng/ml) of complementary RNA (cRNA) or 50 nl after injection of 50 ng (1 ng/ml) of RNA. Groupsof 7–10 oocytes were equilibrated in 700 μl of transport buffer at 0.032, 0.32, 3.2, or 10 μM H$^+$ (pH 7.5, 6.5, 5.5, or 5.0, respectively) for 10 min at 22°C. They were then transferred to 700 μl of transport buffer at 0.032, 0.32, 3.2, or 10 μM H$^+$ containing 50 μM [H$^+$]$_0$-l-[H$^+$] alanine for 1 h at 22°C. Uptake was terminated by rinsing four times in 5 ml of ice-cold buffer. Individual oocytes were lysed in 5% SDS, 2 ml of scintillation mixture was added, and the amount of radioactivity per oocyte was determined.

Electrophysiological Methods—The 2-electrode voltage-damp method was used to measure the kinetics of H$^+$-amino acid cotransport as described previously (Loo et al., 1993; Boorer et al., 1994). Two protocols were used to measure inward currents: 1) The oocyte membrane potential was clamped at −50 mV, and currents induced in response to the addition of amino acids were continuously monitored by a chart recorder. 2) The oocyte plasma membrane was held at −50 mV, and membrane currents were measured after stepping from the holding potential ($V_h$) to test potentials ($V_m$) between −150 and −50 mV in 20-mV increments. Each voltage pulse was applied for 40 ms. The currents were averaged from five sweeps, filtered at 500 Hz, and digitized at 100 μs per point. Steady-state amino acid-induced currents were obtained by taking the difference between steady-state currents at 40 ms in the presence and absence of amino acid. Base-line currents recorded at 0.032 μM H$^+$ (pH 7.5) in the absence of organic substrate were monitored throughout the experiments. Oocytes were reequilibrated at the [H$^+$]$_0$ of the test solution for about 2 min before exposure to amino acids. After every exposure, they were washed in amino acid-free solutions at 0.032 μM H$^+$ until the currents returned to baseline levels.

For kinetic analysis, the amino acid-induced steady-state currents ($i_m$) at each test potential ($V_m$) were fitted to Equation 1 using ENZFITTER software (Elsevier-Biosoft):

$$i = i_{max}(S/S_0 + (K_{binding}))$$

(Eq. 1)

where $i_m$, [amino acid]$_0$, or [H$^+$]$_0$, $i_{max}$ is the maximal current for saturating $S_0$, $K_{binding}$ is the apparent $K_m$ of the substrate ($S_0$ giving half the $i_{max}$), and $n$ is the Hill coefficient.

Substrate Specificity—The substrate specificity of AAP1 was investigated by measuring steady-state substrate-dependent currents with 10 μM of various organic substrates at 10 μM H$^+$. All substrate-induced currents were normalized with respect to the alanine-induced current obtained at −150 mV, which was taken as 100%.

RESULTS

Fig. 1A shows membrane potential changes in an oocyte expressing AAP1 in response to changing [H$^+$]$_0$ and the addition of amino acids. Addition of 1 mM alanine at 10 μM H$^+$ depolarized the oocyte membrane potential by 71 mV (−42 mV to 29 mV). The depolarization induced by 1 mM histidine was less (20 mV). These results indicated that there was a substrate-induced net inward movement of positive charge. Decreasing the [H$^+$]$_0$ from 10 to 0.032 μM and removal of organic substrate from the transport buffer restored the oocyte resting potential. The same oocyte was clamped at −50 mV, and currents were recorded after increasing [H$^+$]$_0$ and adding amino acids to the bathing medium (Fig. 1B). Addition of 1 mM alanine induced an inward current (−250 nA), which returned to baseline levels after washing with alanine-free buffer at 0.032 μM H$^+$. The inward current induced by histidine was less (−45

$^2$The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; HOMOPIPES, homopiperezine-N,N'-bis-2-(ethanesulfonic acid); P face, protoplasmic face.
nA). A membrane potential depolarization (−30 mV) and inward current (−20 nA) were also observed when the [H⁺]₀ was increased from 0.032 to 10 μM in the absence of organic substrate indicating an uncoupled transport of H⁺ through the transporter. These currents were about 10% of the currents induced by 1 mM alanine at 10 μM H⁺. Smaller depolarizations (−18 mV) and inward currents (−5 nA) were also recorded from control oocytes when the [H⁺]₀ was increased from 0.032 to 10 μM. Amino acid-induced depolarizations and currents were not observed in water-injected oocytes. There were no qualitative or quantitative differences in the amino acid-induced currents when chorine in the transport buffer was replaced by Na⁺ or K⁺ or when Cl⁻ was replaced by gluconate.

Fig. 2 shows the current records from a cRNA-injected oocyte, which were obtained by stepping the membrane potential from the holding potential (Vₜₖ = −50 mV) to between −150 and 50 mV in 20-mV increments. At 10 μM H⁺ and in the absence of organic substrate, the currents consisted of an initial capacitive transient, which relaxed to a steady-state level 2 ms after the onset of the voltage pulse (Fig. 2A). Apart from the capacitive transient, no presteady-state cotransporter currents were observed. Inward steady-state currents were induced by amino acids at all applied membrane potentials upon the addition of 10 mM alanine (Fig. 2B). The alanine-dependent current/voltage (I/V) curve (inset) was obtained by subtracting the steady-state currents at 40 ms in the absence of alanine from those recorded in the presence of alanine. Between −150 and 50 mV, the I/V curve exhibited a supralinear dependence on voltage.

To demonstrate that the inward current induced by alanine was accompanied by the uptake of alanine, radiotracer flux experiments were carried out. Table I shows that the uptake of 50 μL L-[³H]alanine in oocytes injected with cRNA was H⁺-dependent, increasing from 14 to 371 pmol/oocyte/h as [H⁺]₀ was increased from 0.032 to 10 μM. The uptake of alanine into water-injected oocytes was H⁺-independent. There was no significant difference in the uptake of alanine between cRNA- and water-injected oocytes at 0.032 and 15.8 μM H⁺.

The kinetics of alanine transport were studied by measuring the steady-state alanine-induced currents (obtained 40 ms after the onset of the voltage pulse) as a function of [alanine] and [H⁺]₀. Figs. 3, 4, and 5 show the data obtained from a single oocyte and are representative of data from three oocytes. Alaneine-dependent I/V curves were obtained at 1.0, 3.2, and 10 μM H⁺ with [alanine]₀ between 50 μM and 20 mM, and at 0.5, 1.0, and 10 mM alanine with [H⁺]₀ between 0.032 and 15.8 μM H⁺.

![Image 156x97 to 457x257](http://www.jbc.org/)

**Fig. 3.** Steady-state current/voltage (I/V) relationships obtained by varying [alanine]₀ at 10 μM H⁺ and [H⁺]₀ at 10 mM alanine. The oocyte membrane was held at −50 mV, and a pulse protocol was used where test potentials ranging from 50 to −150 mV were applied for 40 ms. Currents recorded in the absence of alanine were subtracted from those recorded in the presence of alanine to yield the net alanine-induced steady-state currents. A, I/V curves obtained as a function of [alanine]₀ at 10 μM H⁺. Alanine-induced currents increased supralinearly as the membrane potential was made more negative and increased as [H⁺]₀ increased.

![Image 67x371 to 288x554](http://www.jbc.org/)

**Fig. 2.** Membrane current traces and steady-state I/V relationships obtained at 10 μM H⁺ before and after the addition of alanine to an oocyte injected with AAP1 cRNA. The test potentials shown are −150, −130, −110, −90, −70, −50, and −30 mV and are the average of 5 sweeps. A, current traces recorded in the absence of alanine at 10 μM H⁺. B, current traces recorded after the addition of 10 mM alanine. The inset shows the steady-state alanine-dependent I/V curve obtained at 40 ms by subtracting the steady-state currents in the absence of alanine from those recorded in the presence of 10 mM alanine.

<table>
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**Table I** H⁺ dependence of L-[³H]alanine uptake in oocytes injected with 50 nl (1 μg/μl) AAP1 cRNA or with water

Increasing the [H⁺]₀ from 0.032 μM to 10 μM increased the uptake of L-[³H]alanine into oocytes injected with AAP1 cRNA by 27-fold. Uptake of L-[³H]alanine into water-injected oocytes was low and independent of [H⁺]₀.

TABLE I

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The I/V relationships for alanine at 10 mM H\(^+\) (Fig. 3A) and 10 mM alanine (Fig. 3B) showed a supralinear dependence on voltage between 50 and −150 mV. At 10 mM H\(^+\), the inward currents increased at each membrane potential as the [alanine], increased. Likewise, at 10 mM alanine, the inward currents increased at each membrane potential as the [\(H^+\)]o increased. I/V curves obtained at 1 and 3.2 mM H\(^+\) while varying [alanine], and at 0.5 and 1.0 mM alanine while varying [\(H^+\)]o had the same qualitative characteristics as the I/V curves shown in Fig. 3.

At each membrane potential (−10 to −150 mV) the alanine-induced currents were plotted against [alanine]o or [\(H^+\)]o and the current/concentration curves were fitted to Equation 1. The voltage dependence of the kinetic parameters maximal current for H\(^+\) and [alanine]o decreased, and the Hill coefficient, n for alanine obtained from the fitted data are shown in Fig. 5.

The maximal current induced by alanine \(i_{\text{max}}^\text{alanine}\) exhibited a supralinear dependence on voltage (Fig. 5A) and as shown in Table II, decreased as [\(H^+\)]o decreased. The \(i_{\text{max}}^\text{alanine}\) showed the same voltage dependence (Fig. 5B) and decreased as [alanine]o decreased (Table II). The \(i_{\text{max}}^\text{alanine}\) at saturating [\(H^+\)]o (−1600 nA) was similar to the \(i_{\text{max}}^\text{alanine}\) at saturating [alanine]o (−1400 nA), suggesting a coupling ratio of 1 H\(^+\):1 alanine. Fig. 5C shows that \(K_{\text{a}}^\text{alanine}\) was relatively voltage-independent at 10 mM H\(^+\), became more voltage-dependent as the [\(H^+\)]o decreased, and increased as [\(H^+\)]o decreased (Table III). However, at hyperpolarizing potentials, \(K_{\text{a}}^\text{alanine}\) was almost independent of [\(H^+\)]o. The \(K_{\text{h}}^\text{alanine}\) was slightly voltage-dependent at low [alanine]o, was voltage-independent at 10 mM alanine, and increased as [alanine]o decreased (Fig. 5D; Table III). Therefore, the apparent
affinity for H⁺ and alanine depends on the concentration of their respective cosubstrates. The apparent coupling coefficient n for H⁺ was >1 and was voltage-independent (Fig. 5F). n for alanine was voltage-independent and was >1 over the range of [H⁺]o tested (Fig. 5E).

The substrate specificity of AAP1 was determined by measuring steady-state substrate-induced currents at −150 mV using 10 mM various organic compounds at 10 μM H⁺. Fig. 6 shows the substrate-induced currents normalized with respect to the current induced by 10 mM alanine. AAP1 exhibits a very broad but stereospecific substrate specificity preferring L- to D-isomers. Although there were no inward currents induced by lysine, β-alanine, and 2-methylaminoisobutyric acid at 10 mM, 50 mM of these amino acids induced inward currents, which were 17, 7, and 2% of the alanine-induced current, respectively. The D-isomers of all the amino acids shown in Fig. 6 were tested. Only those that induced an inward current are shown. Other organic compounds that were not transported included sucrose, glucose, malate, and the dipeptide glycyglycine.

To determine whether amino acids differing in their structure and net charge in solution have different transport kinetics, we investigated the kinetics of AAP1 using histidine, glutamate, proline, and glutamine. Fig. 8A shows that the i_{max}^{alanine} > i_{max}^{glutamate} > i_{max}^{glutamine} > i_{max}^{proline} > i_{max}^{histidine}, all of which increased supralinearly with voltage and decreased as [amino acid]o decreased (Table II). The i_{max} values for each amino acid decreased with decreasing [H⁺]o (Table II). The K_{0.5} values for histidine, glutamate, proline, and glutamine at 10 μM H⁺ were voltage-dependent and increased as the membrane potential was made more positive (Fig. 7B) and as [H⁺]o decreased (Table III). The K_{0.5} at 10 mM of each amino acid was voltage-independent (Fig. 7C) and except for proline increased as [amino acid]o decreased (Table III). The current/concentration curves for histidine, glutamate, proline, glutamine, and H⁺ for each amino acid were sigmoidal (data not shown) with Hill coefficients >1.

To obtain an estimate of the number of transporters/oocyte and the turnover number of AAP1, we examined the density of particles on the protoplasmic face (P face) and external face of the plasma membrane of oocytes expressing AAP1 after recording i_{max}^{alanine}. Fig. 8 shows the density of particles on the P face of the plasma membrane of a water-injected control oocyte (A), an oocyte expressing AAP1 (B), and, for comparison, an oocyte expressing the rabbit Na⁺/glucose cotransporter SGLT1 (C). The density of particles in the P face of the control oocyte was 212 ± 43 particles/μm² (Fig. 8A); similar values were obtained for all controls. The density of particles in the P face of the oocyte expressing AAP1 was 1037 ± 136 particles/μm². The capacitance of this oocyte was 297 nF, which corresponds to an oocyte surface area of 2.67 × 10⁻³ μm² (assuming a capacitance of 1 μF/cm²) from which was calculated the total number of particles (2.4 × 10³/particle). The turnover number of AAP1 was calculated using the equation i_{max}^{alanine} = kzeN_T, where i_{max}^{alanine} = maximal current induced by alanine at −150 mV (2712 nA), k = turnover number, z = number of charges per transport cycle (2), e = elementary charge, and N_T = number of transporters per oocyte. These values yield a turnover number of 350 s⁻¹. Turnover numbers of 580 and 800 s⁻¹ were calculated for two other oocytes expressing AAP1. The particle density of the oocyte expressing rabbit SGLT1 (Fig. 8C) was 4121 ± 950/μm², and the i_{max} of 515 nA. This corresponds to a turnover number of 11 s⁻¹. Expression of AAP1 and SGLT1 did not increase the density of particles (~900/μm²) on external faces data not shown.
DISCUSSION

Expression of the H+/amino acid cotransporter (AAP1) from A. thaliana in Xenopus oocytes has allowed us to directly determine the substrate specificity and kinetics of the transporter using electrophysiological, radiotracer flux, and electron microscopic methods. Amino acid transport was H+-driven, and the electrogenicity of the transporter was demonstrated by amino acid-induced depolarizations of the oocyte plasma membrane and amino acid-induced inward currents, which were accompanied by the uptake of amino acids into the oocyte.

AAP1 transports a wide spectrum of amino acids and is specific, preferring L- to D-isomers. Neutral amino acids with short side chains were preferred by the transporter and include cysteine, alanine, leucine, serine, and glycine. Amino acids with longer side chains such as citrulline and methionine and those with a γ-phenyl (phenylalanine), β-amide (asparagine), γ-amide (glutamine), and γ-carboxyl (glutamate) were also transported. AAP1 did not so readily transport amino acids containing an imidazole side chain (histidine, β-poly- and cyclic amino acids (proline and hydroxyproline). The basic amino acids arginine, ornithine, and lysine, which have δ-guanido, δ-amino groups, and an ε-amino group, respectively, were transported poorly. The α-amino group is important for substrate recognition; β-alanine and 2-methylamino isobutyric acid were transported poorly, and γ-aminoacetic acid was not. These observations confirm and extend results obtained by expression of AAP1 in yeast (Frommer et al., 1993; Hsu et al., 1993; Kwart et al., 1993; Fischer et al., 1995).

A detailed kinetic investigation of AAP1 was undertaken using alanine, glutamate, glutamine, histidine, and proline, which differ either in their structure and/or net charge in solution. All induced inward currents through the transporter, the magnitude of which increased as [H+]o and [amino acid]o increased and as the membrane voltage was made more negative. This is a direct demonstration that AAP1 operates via a H+-coupled transport mechanism. The transporter exhibited saturation kinetics with respect to [H+]o and [amino acid]o, but not to voltage. Amino acid-induced currents may saturate with respect to membrane voltage in plants where the membrane potential that the transporter senses is probably more negative than −150 mV. For example, in a study of nitrate transport in Arabidopsis roots, the cell-resting potential was −200 mV, and I/V curves were supralinear at −150 mV and did not saturate until −250 mV (see Fig. 5B) (Meharg and Blatt, 1995).

The net charge of amino acids in solution depends on the pKas of their side groups. Greater than 99% of alanine, proline, and glutamine in solution are zwitterions over the range of [H+]o (0.032–10 μM) used in the experiments presented here. About 95, 24, and 9% of histidine and 0.06, 5, and 15% of glutamate are zwitterions at 0.032, 3.2, and 10 μM H+o, respectively; the majority of the other charged species of histidine and glutamate bear a single positive charge or a single negative charge, respectively. In view of the structural and charge differences between these amino acids, it is interesting that the kinetic properties of the transporter for each amino acid were qualitatively similar.

1) The current/voltage curves did not saturate at negative membrane potentials. 2) The I max decreased as [H+]o decreased. 3) The I max for each amino acid decreased as [histidine]o increased. 4) The [amino acid]o was voltage-dependent and decreased as the membrane potential was made more negative. 5) Except for proline, the [amino acid]o was voltage-dependent and became slightly more voltage-dependent at lower [amino acid]o.

We propose a kinetic model of amino acid transport by AAP1 (Fig. 7). In this mechanism, 2 H+o 2 substrate molecules (S) bind to the transporter C1 at the external face of the membrane to form CH2S1 or CS2. These intermediates then bind S2 or H2 respectively, to form the complex CH2S2. A conformational change allows H2 and S2 to be transported to the cytoplasmic surface where they dissociate. The transport cycle is completed when the empty transporter C1 undergoes another conformational change allowing the ligand binding sites to face the external surface. The broken lines between CH2S1 and CH2S2 indicate possible uncoupled transport of H+. The rationale for this mechanism is as follows. The H+ and amino acid activation curves for all the amino acids tested were sigmoidal with Hill coefficients for H+ and amino acids >1. To simplify the transport model, we assume that the Hill coefficients for H+ and amino acid are 2. Thus, we propose that 2 H+ and 2 substrate molecules bind to the transporter per transport cycle. The stoichiometry of transport could not be determined from reversal potentials as the currents did not reverse over the voltage range used in the experiments. Uncoupled transport of H+ is proposed based on the observation that increasing [H+]o in the...
absence of amino acids induced inward currents that were significantly higher than those induced in water-injected controls; the depolarizations observed in control oocytes are due to the $H^+$ inhibition of an outward $K^+$ current (Burckhardt and Fromter, 1992). However, without a specific blocker of amino acid transport, we are unable to draw conclusions from this observation in the present study. Uncoupled transport of $Na^+$ occurs through the $Na^+/glucose$ cotransporter (Umbach et al., 1990; Parent et al., 1992a, 1992b) and the serotonin transporter (Mager et al., 1994). There is no significant leak for amino acid through AAP1; flux experiments showed that at low $[H^+]_o$, there was no uptake of amino acid above water-injected controls.

A random versus an ordered, and a simultaneous versus a sequential mechanism of transport, are evaluated using the kinetic parameters obtained with alanine. Fig. 10 shows a replot of the data presented in Fig. 6. The $K_{i\text{max}}^{\text{alanine}}$ decreased at all membrane potentials as $[H^+]_o$ became saturating (Fig. 10A). Likewise, the $K_{i\text{max}}^{\text{alanine}}$ decreased as $[alanine]_o$ became saturating (Fig. 10B). The increase in the apparent affinity of the transporter for $H^+$ and amino acid as the concentration of their respective cosubstrates increased suggests positive cooperativity between both ligands for binding to their respective sites on the transporter. This cooperativity suggests that both ligands must be bound to the transporter before the transporter-$H^+$-amino acid complex crosses the membrane, i.e. that the transporter operates via a simultaneous mechanism (J auch and Laüger, 1986). The $i_{\text{max}}^{\text{alanine}}$ increased as $[H^+]_o$ increased at all membrane potentials (Fig. 10C), and $i_{\text{max}}^{\text{alanine}}$ increased as $[alanine]_o$ increased (Fig. 10D). The $i_{\text{max}}$ for both $H^+$ and amino acid was dependent on the concentrations of their respective cosubstrates, which would suggest a random binding of substrates to the transporter (J auch and Laüger, 1986). Results presented in Tables II and III suggest that the same mechanism applies to all the amino acids tested. There was no decrease in $K_{i\text{max}}^{\text{alanine}}$ when [proline] was increased from 1 to 10 mM, probably because 10 mM proline is not saturating.

AAP1 transports neutral basic, and acidic amino acids. The $i_{\text{max}}^{\text{alanine}} > i_{\text{max}}^{\text{glutamine}} > i_{\text{max}}^{\text{glutamate}} > i_{\text{max}}^{\text{proline}} > i_{\text{max}}^{\text{histidine}}$ and the shape of the I/V curves are the same. This suggests that the mechanism of transport for each amino acid is the same and that differences in $i_{\text{max}}$ are due to changes in a rate-limiting step in the transport cycle, possibly the translocation rate of the fully loaded transporter ([CH$_2$S$_2$O]$_o$) or any of the dissociation steps on the inside of the cell. These rates cannot be determined from measurement of inward currents in intact oocytes. The I/V curves did not saturate between −150 and 50 mV, and this was independent of $[H^+]_o$ and $[alanine]_o$. Therefore, at least one
Kinetics of AAP1 Expressed in Oocytes

The turnover number calculated for AAP1 is higher than that obtained for other cloned cotransporters. Using electrical methods, a turnover number of 59 s\(^{-1}\) was determined for the H\(^+\)/hexose cotransporter (STP1) (Boorer et al., 1994), 60 s\(^{-1}\) for human SGLT1 (Loo et al., 1993), and 25 s\(^{-1}\) for rabbit SGLT1 (Panayotova-Heiermann et al., 1994).

In conclusion, transport by AAP1 is relatively fast compared to other cloned cotransporters and is H\(^+\)-dependent and electrogeneic with membrane voltage enhancing the maximal transport rate and the affinities for H\(^+\) and amino acid. Future kinetic studies of other members of the AAP gene family, in conjunction with site-directed mutagenesis of AAP1, will enable us to identify the amino acid residues involved in substrate and H\(^+\) binding and in voltage regulation.

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REFERENCES


Rate-limiting step in the transport mechanism is potential-dependent over this voltage range. There is a voltage dependence of \( K_{\text{app}} \) and \( K_{\text{app}} \) amino acid at low concentrations of amino acid and H\(^+\), respectively, which decreases as the concentration of ligands increases. Therefore, part of the effect of voltage is to enhance the affinity of the transporter for substrates. Even at saturating [H\(^+\)] and [amino acid], there is a voltage dependence of \( i_{\text{max}} \). Therefore, reaction steps other than H\(^+\) and amino acid binding are voltage-dependent, possibly the conformational changes of the empty and loaded transporters.

Previous studies have shown that neutral, basic, and acidic amino acids are transported by different mechanisms within the plant. For example, in Chlorella (Cho and Komor, 1983) and sugar cane cells (Wyse and Komor, 1984), neutral amino acids were accompanied by an influx of H\(^+\) and an efflux of K\(^+\). Wyse and Komor (1984) observed that basic amino acids were driven by the negative membrane potential and were not cotransported with H\(^+\) and that acidic amino acids were accompanied by the uptake of 2 H\(^+\) and the release of K\(^+\). Kinrade and Etherton (1980) obtained similar results with amino acid transport in oat coleoptiles. Unlike plant cells where the interpretation of amino acid transport data is complicated by the presence of a variety of transport systems, overexpression of AAP1 in oocytes also increased the density of transport proteins including the Na\(^+\)/glucose cotransporter from rabbit small intestine (SGLT1) and the water channels MIP and Etherton (1980) obtained similar results with amino acid transport data is complicated by the presence of a variety of transport systems, overexpression of AAP1 in oocytes also increased the density of transport proteins including the Na\(^+\)/glucose cotransporter from rabbit small intestine (SGLT1) and the water channels MIP and STP1 (Panayotova-Heiermann et al., 1994).

The expression of cloned transporters increased the density of both substrates. This turnover number is an underestimate because the \( i_{\text{max}} \) did not saturate with respect to membrane voltage. The turnover number calculated for AAP1 is higher than that obtained for other cloned cotransporters. Using electrical methods, a turnover number of 59 s\(^{-1}\) was determined for the H\(^+\)/hexose cotransporter (STP1) (Boorer et al., 1994), 60 s\(^{-1}\) for human SGLT1 (Loo et al., 1993), and 25 s\(^{-1}\) for rabbit SGLT1 (Panayotova-Heiermann et al., 1994).

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Kinetics and Specificity of a H/Amino Acid Transporter from *Arabidopsis thaliana*
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