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MECHANISM OF PROLINE UPTAKE BY *CHLORELLA VULGARIS*

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An amino acid uptake system specific for glycine, alanine, serine and proline was induced by glucose in *Chlorella vulgaris*. The uptake system translocated the zwitterionic form of the amino acid. There was more than 100-fold accumulation which indicated a coupling to metabolic energy. The depolarization of the membrane potential during proline uptake and the sensitivity of its uptake rate to the membrane potential point to coupling with an ion flow. Inhibitors of plasmalemma-bound H^+-ATPase inhibit proline uptake. These data are interpreted to mean that proline is taken up as a proton symport. In some *Chlorella* strains the proline-coupled H^+ uptake could be measured with electrodes, but not in *Chlorella vulgaris*. There is evidence that the transport of amino acids rapidly stimulates the proton-translocating ATPase of *Chlorella vulgaris*, so that the proline-coupled proton uptake is immediately neutralized.

Introduction

The green alga *Chlorella vulgaris* has a glucose uptake system which had been studied in the past as a model system for sugar uptake mechanism in plants [1–3]. The sugar transport system can be induced by D-glucose [4,5]. Recently it was found that preincubation of *Chlorella* cells with D-glucose not only induces the glucose transport system but also dramatically increases the activities of two distinct amino acid transport systems, one specific for basic amino acids, the other for short-chain neutral amino acids [6]. Some properties of these amino acid uptake systems are different from the sugar uptake system. For instance, the plateau level of amino acid accumulation is reached because the high concentration of intracellular amino acid inhibits the influx [7], whereas the plateau level of sugar accumulation is achieved because the influx is balanced by efflux [8]. It was of interest, therefore, to study in more detail the mechanism of amino acid uptake with respect to its coupling to metabolic energy. In sugar transport, a proton-coupled transport had been found [2], and proton-coupled amino acid uptake in higher plants [9] and sodium-coupled amino acid uptake in animals [10] had been reported.

Material and Methods

The green alga *Chlorella vulgaris* was grown autotrophically on inorganic salt medium with CO_2-enriched air and continuous light [1]. The cells were harvested by centrifugation at $5000 \times g$ for 10 min and incubated for 3 h in 1.4 mg/ml D-glucose in 25 mM sodium phosphate, pH 6.0 at 25 μl packed cells per ml. This incubation induced the amino acid uptake systems. The incubation medium for uptake experiment normally contained 25 mM sodium phosphate, pH 6.0, 6 μl packed cells per ml with 1 mM amino acid. labelled with ^{14}C at a specific activity of 0.5 Bq/nmol. For K_m determination, lower cell densities were used. 0.5 ml samples were withdrawn at 30-s intervals, rapidly filtered on membrane filters (0.8 μm pore size, Schleicher-Schüll GmbH, Dassel, F.R.G.) and

radioactivity detected by scintillation spectrometry without prior extraction.

The flux of ions such as H^+, K^+ etc. into the cells was followed in a small chamber containing a mechanical stirrer and inserted electrodes. The electrode potential was amplified and continuously recorded. The composition of the incubation medium was such that the ion to be determined was at low concentration in the medium. In the case of pH measurement, the cells were incubated without buffer. The ion flux before and after addition of 1 mM amino acid was followed. For stoichiometry determination, labelled amino acid was added as for the uptake experiments and samples were withdrawn out of the chamber.

The membrane potential was determined by the distribution of [^{3}H]tetraphenylphosphonium chloride or as the flux rate of [^{3}H]tetraphenylphosphonium [1].

The strain of *Chlorella vulgaris* was the same as used previously [1]. The other strains of *Chlorella* were from Algensammlung (Göttingen). The labelled amino acids were from New England Nuclear (Colnbrook, U.S.), the analogues of proline were from Aldrich Chemicals (Nettetal), and Sigma (München).

Labelled tetraphenylphosphonium was a gift of Dr. P. Geck (Frankfurt). The pH-electrode was from Ingold (Frankfurt), the K^+, Na^+, Ca^{2+} and Cl^--specific electrodes were from Philips (Hamburg).

Results

Specificity of the uptake system

The uptake system was previously found to be induced after glucose preincubation. The uptake rates of four neutral L-amino acids, glycine, alanine, serine and proline were increased. The relative affinity of the transport system for these four amino acids differed by a factor of 10 (Table I). Alanine and proline seemed to be the best substrates. Serine and glycine were of lower affinity. α-Aminoisobutyric acid and D-alanine had no affinity for the transport system. The amino acids were rapidly metabolized by the cells with the exception of proline, which remained more than 70% nonmetabolized. Proline was, therefore, usually used as the substrate for the uptake system and the uptake system is for convenience called the proline uptake system.

TABLE I

K_m VALUES FOR UPTAKE OF NEUTRAL AMINO ACIDS

The buffer used was 25 mM sodium phosphate, pH 6.0.

	K_m (μM)
Glycine	201
Alanine	77
Serine	769
Proline	106

Since the pK for protonation of proline is 2.0 and 10.6, the K_m for the uptake of proline should remain nearly constant over the pH-range of 4 to 8, if the neutral form of proline is taken up. Fig. 1 shows that there is a slight variation of K_m from pH 4 to 7, but much less than would be expected if a charged form was taken up (in that case a 10-fold change in K_m per pH unit would be expected). Thus it is the neutral form of proline which is transported. This was substantiated by a series of competition studies with analogues of proline. All the analogues with a negatively charged carboxyl group and a positively charged nitrogen in the ring competed with proline. Those molecules with oxygen or sulfur instead of nitrogen in the ring, or with a carbonyl group instead of carboxyl group, did not compete with proline (Fig. 2). In addition, the molecules which do not bear the correct charge did not compete even when the ring structure is the same as that of proline, e.g. in 5-pyrrolidone-2-carbonic acid (where the keto group changes the pK of the nitrogen so that there is no protonation at neutral pH). Analogues of

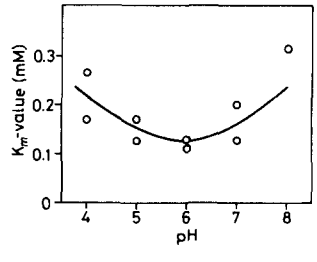

Fig. 1. K_m for proline uptake at different pH. The medium contained 25 μM citrate-phosphate buffer, the cell density was 5 μl packed cells/ml.

competing analogues	K_i (mM)	pK	not competing analogues	pK
pyrrole-2-carbonic acid	11.0	4.8 12.5	prolinol	9.5
piperidine-2-carbonic acid	3.8	2.0 10.6	furane-2-carbonic acid	3.2
L-thiazolidine-4-carbonic acid	0.16	1.4 6.3	thiophene-2-carbonic acid	3.5
L-2-acetidine carbonic acid	0.27	2.0 10.6	pyrrolidone	–
			L-5-pyrrolidone-2-carbonic acid	3.3

Fig. 2. Proline analogues which compete with proline and those which do not.

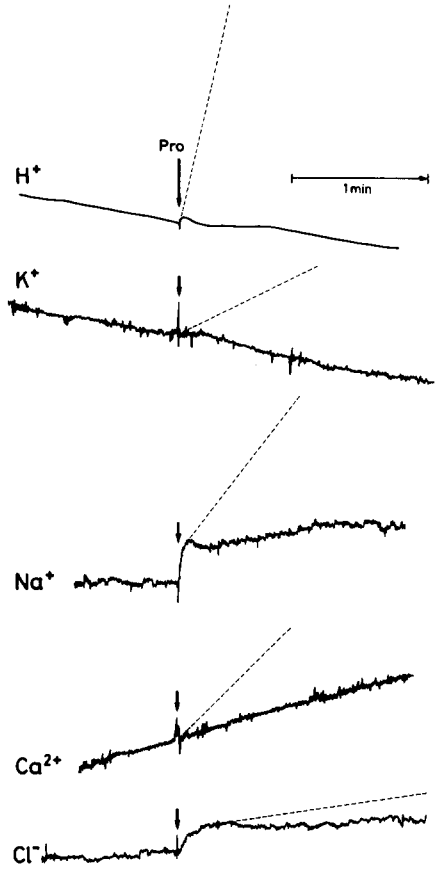

Fig. 3. Ion concentration in the medium before and after addition of proline. The H^+ and K^+ flux recordings were performed in 5 mM NaCl, the Na^+ measurement was in 5 mM KCl, the Cl^- measurement in 5 mM Na_2SO_4.

alanine such as L-lactic acid had no effect on alanine or proline uptake.

Is proline taken up by secondary active transport system?

Proline can be accumulated more than 200-fold [7]. If proline uptake is coupled to the flow of an ion, it should be detectable using ion-selective electrodes. However, neither H^+, Na^+, Ca^{2+} nor Cl^- flux was found to occur along with proline uptake (Fig. 3), even though the limit of detection would have been sufficient if a one to one stoichiometry of ion and proline flux existed (dotted line). It can be argued that another ion than those measured is co- or countertransported, but then at least the charge compensating outflow of K^+ or H^+ should be seen as for example in arginine uptake (Cho, B.-H., unpublished data). Furthermore, if an efficient coupling to ion flow occurs then it should be a more abundant ion and not for instance a trace element. Also K^+, Na^+ and Cl^- did not stimulate proline uptake.

Is the plasmalemma ATPase involved in proline transport?

The effect of inhibitors of the proton translocating ATPase, such as diethylstilbestrol and vanadate, and of protonophores on proline uptake was measured. As shown in Fig. 4 proline transport was inhibited by dicyclohexylcarbodiimide and carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP), and also by diethylstilbestrol and vanadate which are thought to be specific inhibitors of ATPase with phosphorylated intermediates such as the H^+-ATPase at the plasmalemma. Proline uptake was inhibited to exactly the same extent as was uptake of 6-deoxyglucose which is known to be transported via proton symport [2]. These data suggest an involvement of the H^+-translocating ATPase despite the negative results with the pH-recordings.

Role of membrane potential in proline transport

Since it had been shown that the neutral form of proline is taken up (Fig. 2), the transport should be without effect on membrane potential if no coupled ion flow occurs. The addition of proline

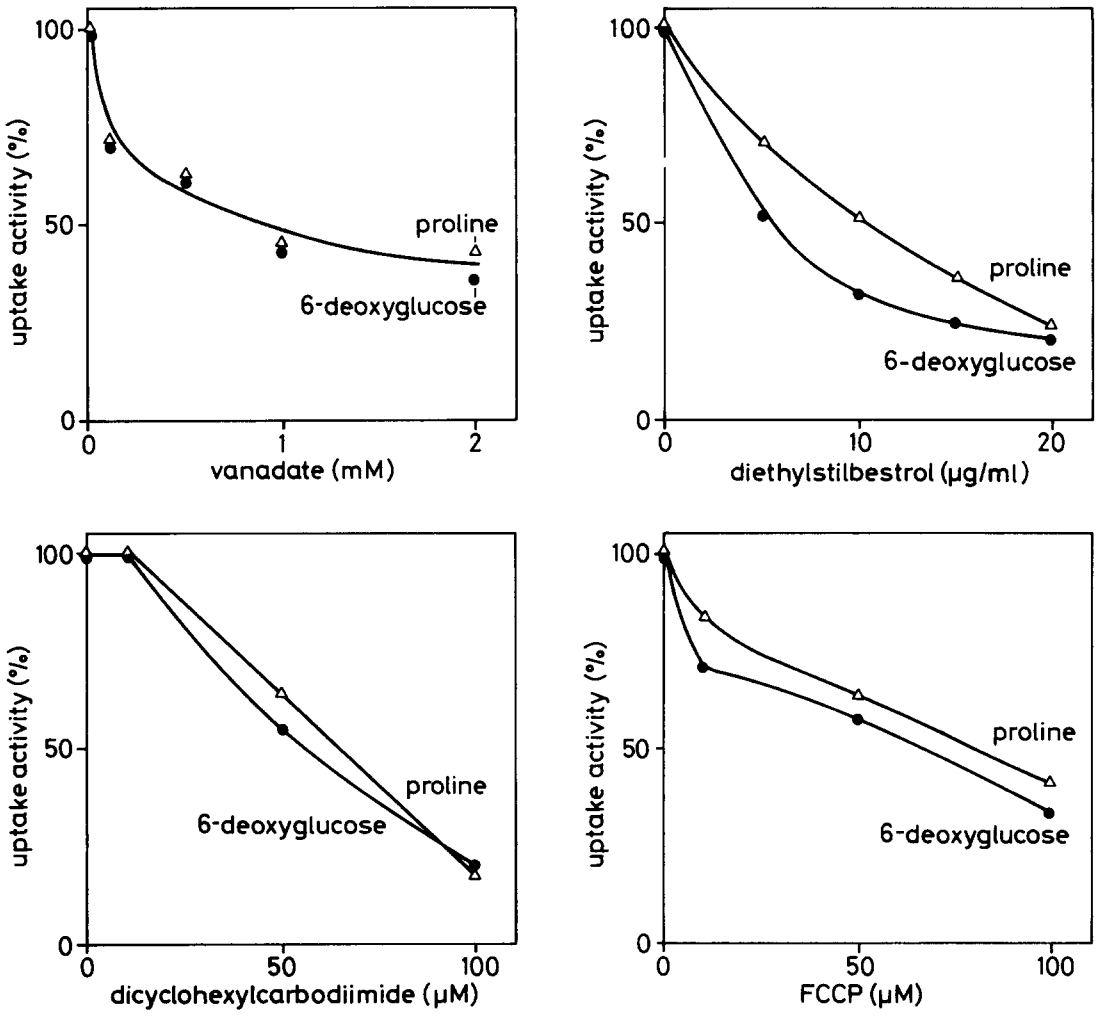

Fig. 4. Effect of inhibitors and uncoupler on proline uptake. For the vanadate experiment 25 mM Hepes-Tris buffer, pH 6.0 was used.

depolarized the membrane to a small but significant extent. In addition an artifical reduction of the membrane potential decreased the rate of proline uptake (Table II). The membrane potential can also be decreased by the addition of 6-deoxyglucose because its transport is coupled to proton influx. 6-Deoxyglucose reduces proline uptake in a non-competitive manner (Fig. 5). The obvious explanation is that 6-deoxyglucose and proline use the same energy source.

Does proline stimulate the H^+-ATPase?

Circumstantial evidence, such as inhibitor sensitivity and membrane depolarization, suggest that proline transport is coupled to influx of positive ions (probably protons). It can be argued that the proton uptake together with proline is obscured because proline immediately stimulates the H^+-

TABLE II

RELATIONSHIP BETWEEN PROLINE TRANSPORT AND MEMBRANE POTENTIAL

	Change of membrane potential (mV)	Proline uptake (μmol/h/ml packed cells)
with proline	10±1	98
with 30 mM KCl and proline	30±6	67

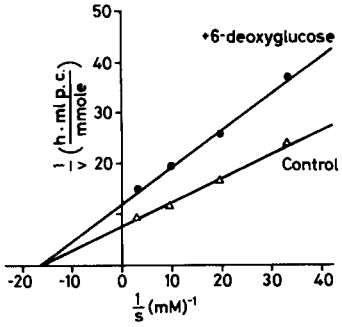

Fig. 5. Effect of 6-deoxyglucose on proline transport. The concentration of 6-deoxyglucose was 1 mM.

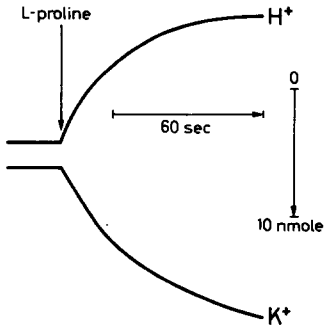

Fig. 6. H^+ and K^+ flux with proline of *Chlorella* 211-8e. For H^+ and K^+ flux recordings the medium contained 5 mM NaCl.

ATPase so that the pH-change in the medium is too small to be picked up by the pH electrode. If this was true then the 6-deoxyglucose-induced transient proton uptake should be reduced by the previous proline addition and in fact was this the case (Table III). The stoichiometry of H^+ per 6-deoxyglucose molecule was significantly reduced by the addition of proline a few seconds before adding 6-deoxyglucose. Thus, there is a possibility that proline is transported together with protons and that it is merely the fast compensatory proton ejection of the H^+-ATPase which obscures that proton movement. Another strain of *Chlorella* did not have such a complete compensatory ejection of protons. This strain, 211-8e, showed a proline specific H^+-uptake and a charge compensating K^+ outflow (Fig. 6). The stoichiometry was 0.3 H^+/ proline molecule. The observed 210-fold accumulation of proline [7] could be brought about easily by the protonmotive potential difference across the plasmalemma [11].

TABLE III

EFFECT OF PROLINE ON H^+/6-DEOXYGLUCOSE STOICHIOMETRY

The concentration of proline and 6-deoxyglucose was 1 mM each.

Condition	Stoichometry (H^+/6-deoxyglucose)
Control	0.68
Proline at the same time as 6-deoxyglucose	0.71
Proline 10 s before 6-deoxyglucose	0.49
Proline 30 s before 6-deoxyglucose	0.42
Proline 60 s before 6-deoxyglucose	0.39

Discussion

The characterized amino acid uptake system is specific for the short-chain neutral L-amino acids glycine, alanine, serine and proline. The narrow specificity is comparable to the specificity of amino acid uptake systems in fungi and bacteria [12–14] and is in contrast to the amino acid uptake systems in higher plants, where all neutral amino acids are taken up by one system [15]. The control of steady-state transport, namely the continuous slowing-down of the influx reaction by increasing internal amino acid levels [7] is also very similar to fungal amino acid uptake systems. The neutral amino acids are taken up in the zwitterionic form and therefore analogues which lack one of the charges are not accepted by the transport system. This phenomenon is not unusual since the charge of the substrate normally plays a decisive role in the binding [16]. It is interesting to note the decrease of proline uptake by membrane depolarization whereas hexose uptake rate is insensitive to membrane potential [17].

The search for the mechanism of proline uptake was complicated. There was evidence from membrane potential studies that coupled ion flow occurs, but no ion flow was found with electrodes in *Chlorella vulgaris*. Finally a few other *Chlorella* strains showed some proline-proton-symport, although at a low stoichiometry. The reason for the

stoichiometry in *Chlorella vulgaris* being below the measuring limit, is probably a very fast stimulation of the proton-ejecting ATPase. Such a fast compensating proton ejection had been found in the mechanism of sugar uptake by *Neurospora* [2] so that a proton symport was hardly detectable. An overcompensating proton efflux is reported for yeast [12], so that a net acidification of the medium is observed, despite proton symport with lysine. The mechanism of H^+-ATPase stimulation is however unclear. It is possible that the amino acids directly stimulate the ATPase, but the amount of intracellular proline increases by only 3% during the first 10 s of the H^+-flux measurement, so that a drastic effect of that small change of concentration seems unlikely. If the intracellular amino acids are, however, sequestered in organelles then the increase of cytoplasmic proline could be more significant.

Another possibility is that there is a fast-responding fraction of H^+-ATPase and a slow-responding fraction. Evidence for differently active fractions of H^+-ATPase has been reported for liver mitochondria [19] and chloroplasts [20]. Since the proline uptake rate is about one quarter that of hexose uptake, the fast-responding fraction of H^+-ATPase might just be sufficient to compensate the prolin-coupled proton inflow, whereas hexose-coupled proton inflow needs the slow-reacting fraction of H^+-ATPase, so that for at least 30 s a proton transient can be observed. This hypothesis is, however, not substantiated by the measurement of H^+/6-deoxyglucose stoichiometry after previous proline addition: It is expected that the fast ATPase fraction is exhausted by proline transport and the stoichiometry of 6-deoxyglucose-induced proton transient therefore increases, but in reality it decreases. An alternative explanation may be afforded by the following: why for instance, is a proton transient observed with hexoses? Why does the ATPase not immediately compensate for the inward flow of protons? Further studies on regulation of plasmalemma ATPase are therefore needed.

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