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Effect of arginine modification on K^+-dependent leucine uptake in brush-border membrane vesicles from the midgut of *Philosamia cynthia* larvae

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Abstract

The effect of phenylglyoxylation on the midgut K^+-dependent leucine transport was studied using lepidopteran brush-border membrane vesicles. The inhibition of leucine uptake by phenylglyoxal (PGO) showed a biphasic inactivation pattern. The second-order rate constant for the slow and fast phases were 0.0020 mM^{-1} min^{-1} and 0.0091 mM^{-1} min^{-1}, respectively. However, substitution of borate buffer for Hepes-Tris buffer produced a mono-exponential inactivation pattern, suggesting modification of a single arginine group. The effect of PGO was dose-dependent and the concentration causing half-maximal inhibition of leucine uptake was 5.1 ± 0.3 mM. Leucine transport was significantly inhibited also in the absence of a potassium electrochemical gradient (i.e., $[K^+]_{in} = [K^+]_{out} = 100$ mM), suggesting that inhibition was not related to a decrease in the driving force. Moreover, intravesicular volume remained unchanged after preincubation with PGO. Kinetic analysis of the interaction of PGO with the leucine cotransporter revealed that (i) inhibition was related to a decrease in the V_{max} value and (ii) neither leucine nor K^+ were able to prevent the inhibition. Our results suggest an important role for arginine residues in the molecular mechanism of K^+/leucine cotransport in lepidopteran larvae midgut.

Key words: Phenylglyoxal; Potassium ion/leucine cotransport; Leucine transport; Brush-border membrane vesicle; Chemical modification; Lepidopteran larva

1. Introduction

The midgut of lepidopteran larvae absorbs amino acids through a secondary active process coupled to a K^+ electrochemical gradient [1–4], maintained in vivo by a an electrogenic vacuolar-type ATPase associated to a K^+/H^+ antiporter [5,6]. The striking difference between this transport agency and the typical Na^+-dependent cotransporter described in vertebrates is the lack of cation specificity, so that both K^+, Na^+ and in some instances Li^+ are all able to activate amino acid transport [3,7]. This peculiarity may be responsible for the different kinetic mechanism involved in leucine translocation across lepidopteran brush-border membranes [8].

Chemical modification of proteins by specific reagents has been a useful tool in the structural and functional characterization of carriers [9–14]. Many studies directed towards a search for catalytically important residues associated with the molecular mechanism of Na^+-dependent translocation of organic compounds across mammalian brush-border membranes, have led to the formulation of plausible models of operation of both cotransporters and antiporters [13,14]. Thus, as far as the Na^+/glucose cotransporter is concerned, a carboxyl group and (an) amino group(s) are very likely involved in the binding of glucose [12,15], whereas tyrosine residues are very close to the sodium binding site [10,16]. A carboxyl group is also involved in the binding of Na^+ to the Na^+/H^+ exchanger [13]. Other residues, such as thiol groups [11,14], histidine [9,17,18] and arginine residues [19,20] are probably not

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Abbreviations: BBMV, brush-border membrane vesicles; PGO, phenylglyoxal; TMACl, tetramethylammonium chloride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid.

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involved in the binding sites for amino acids or sugars but may play a role in the translocation step.

It has been demonstrated that highly reactive arginine residues are involved in different cotransport mechanism in mammalian kidney [19–21]. The purpose of this study is to evaluate the sensitivity of lepidopteran K^+/leucine cotransport to modification by PGO. The results here presented are the first demonstration that catalytically important guanidinium groups are involved in the operation of the carrier, suggesting structural similarities with the Na^+-driven mammalian cotransporters.

2. Materials and methods

Materials. L-[4,5-^{3}H]Leucine 152 Ci/mmol was purchased from Amersham International, Amersham, UK. TMACl was from Sigma (St. Louis, MO). PGO was purchased from Aldrich. It was recrystallized from hot water and stored dessicated in the dark at 4°C. Stock solutions were prepared daily in the appropriate buffer and PGO concentration was determined spectrophotometrically at 250 nm using an ϵ value of 12600 mM^{-1} cm^{-1} in H_2O. All other reagent were analytical grade products from Merck (Darmstadt, Germany).

Animals. Fifth instar larvae of *Philosamia cynthia* were reared in the laboratory and fed on *Ailanthus glandulosa* leaves. The larvae were chilled on ice and midguts excised, rinsed, rapidly frozen in liquid nitrogen and then stored in liquid nitrogen as described elsewhere [22].

Membrane preparation and transport studies. BBMV were prepared from frozen midguts as previously described [2]. Unless otherwise stated, the final pellet was resuspended in 100 mM mannitol, 10 mM Hepes-Tris (pH 7.4) (HT buffer) at a protein concentration of 1–5 mg/ml, as assayed according to Bradford [23], using bovin serum albumin as standard.

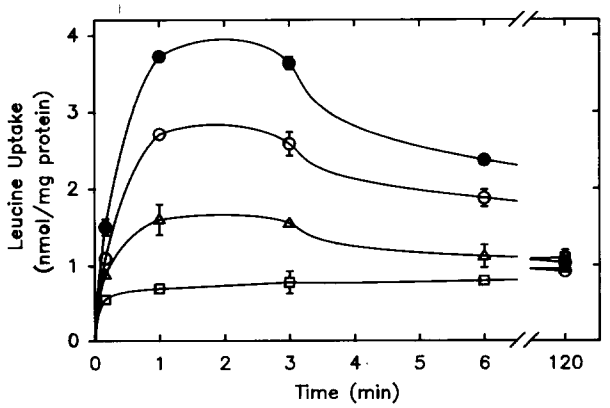

Fig. 1. Effect of PGO on leucine uptake. Vesicles, at a protein concentration of 1.87 mg/ml, were preincubated in the presence of the following PGO concentrations (mM): 47 (□), 20 (△), 5 (○) and 0 (●). After extensive washing of the vesicles, uptakes were determined at the indicated times, as described in Materials and methods. Data represent a typical experiment performed in triplicate. When absent, S.E. bars were smaller than the symbols used.

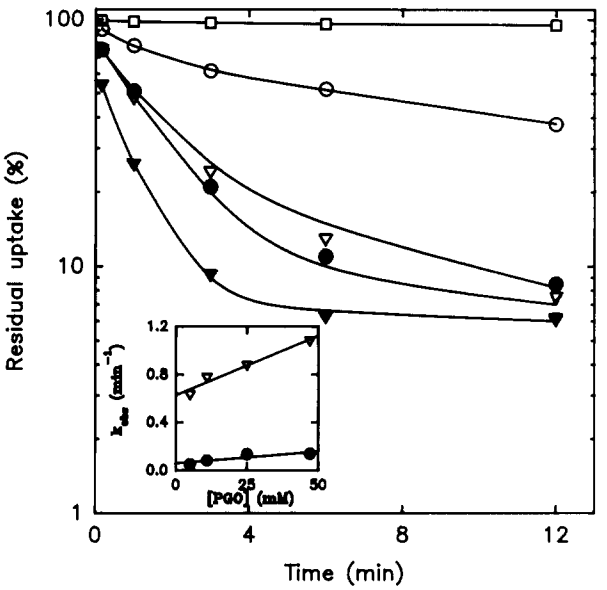

Fig. 2. Rate of PGO inactivation of leucine uptake. Vesicles, at a protein concentration of 2.5 mg/ml, were preincubated for the indicated period of time in the presence of the following PGO concentrations (mM): 47 (▼), 25 (●), 11 (▽), 5 (○) and 0 (□). Uptakes were determined after 12 s of incubation and plotted with respect to the uptake at 0 min of preincubation. Data were fitted to a sum of two exponential decays and represent a typical experiment performed in triplicate. In the inset, the k_{obs} calculated for the slow (●) and the fast (▽) phases were plotted as a function of PGO concentration.

Transport experiments were performed in triplicate at 22°C by a rapid filtration technique [2]. Vesicles, either untreated or preincubated with the indicated PGO concentrations, were incubated in HT buffer containing 10 μCi/ml of ^{3}[H]leucine, 0.2 mM leucine and 100 mM KSCN. Kinetic analysis was performed by varying the amino acid concentration from 0.05 to 2 mM and the KCl concentration from 0 to 150 mM (150–0 mM TMACl).

Pre-incubation of membrane vesicles with PGO. Membrane vesicles, at the protein concentration indicated, were incubated at 25°C for various periods of time with different PGO concentrations as reported in the legends of the figures. After incubation vesicles were washed with 100 volumes of HT buffer, centrifuged at 49000 × g for 20 min and resuspended in HT buffer. Uptakes were then performed as indicated above.

Calculations. Kinetic parameters were calculated using a mutliparameter, iterative, nonlinear regression program based on the Marquardt-Levenberg algorithm (SigmaPlot, Jandel, CA).

3. Results

In the presence of an inwardly directed K^+-gradient, brush-border membrane vesicles from *P. cynthia* larvae are able to accumulate leucine 4–5-times over the equilibrium value (see Ref. [24] and Fig. 1). When membranes were preincubated with PGO concentrations ranging from 5 to 47 mM for various periods of time, leucine initial uptake rate, estimated at 12 s, and accumulation of the amino acid were strongly inhibited (Fig. 1). The inhibition of leucine uptake is a time- and a concentration-dependent process. The inactivation curves were biphasic at all PGO concentrations (Fig. 2). When they were analyzed as the sum of two exponential processes, the rates of inactivation obtained for the slow and the fast phases of reaction were both linearly dependent upon PGO concentration, and second-order rate constants of 0.0020 mM^{-1} min^{-1} and 0.0091 mM^{-1} min^{-1}, respectively, could be calculated (Fig. 2, inset). When borate buffer was substituted for HT buffer the inactivation pattern followed a monophasic behaviour (Fig. 3). Under these experimental conditions the calculated k_{obs} in the presence of 5 and 10 mM PGO were 0.037 min^{-1} and 0.066 min^{-1}, respectively. These values were not statistically different from those obtained for the fast phase in Hepes-buffered solutions.

The concentration of PGO which caused 50% inhibition of leucine uptake (IC_{50}) was calculated from a dose–response curve obtained at a fixed preincubation time (15 min) in the presence of different PGO concentrations (Fig. 4). The obtained value was 5.1 ± 0.3 mM.

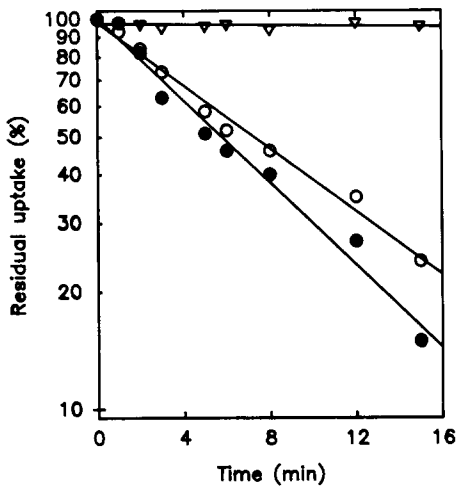

Fig. 3. Rate of PGO inactivation of leucine uptake in borate buffer. Vesicles, at a protein concentration of 3.5 mg/ml, were preincubated as indicated in Fig. 2, except that preincubation was performed in borate buffer (H_3BO_3 100 mM, KOH 63 mM, mannitol 100 mM (pH 7.5)). Symbols represent: (▽) control value in the absence of PGO, (●) 11 mM PGO and (○) 5 mM PGO. Uptakes were determined after 12 s of incubation and plotted with respect to the uptake at 0 min of preincubation. Data represent a typical experiment performed in triplicate.

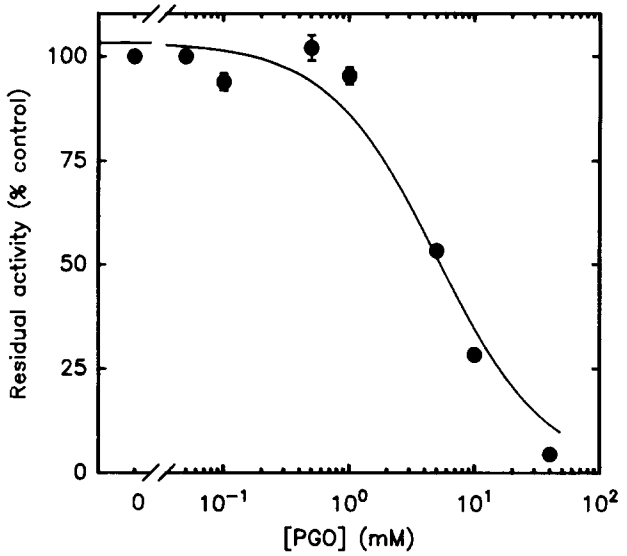

Fig. 4. PGO inactivation of leucine uptake. Leucine uptake was performed at 12 s of incubation time in Hepes-buffered solution, after preincubating the vesicles, at a protein concentration of 5.5 mg/ml, for 15 min at the indicated PGO concentrations. Data were expressed as a percent of the control in the absence of inhibitor and fitted to the equation: $Y = M \cdot IC_{50}/(IC_{50} + [PGO])$, where Y and M are the residual activity in the absence of PGO and at the IC_{50} value, respectively. Data represent a typical experiment performed in triplicate.

The physical integrity of the vesicles after treatment with PGO was evaluated by calculating total intravesicular volume, as the ratio between leucine uptake values at equilibrium (120 min of incubation) and leucine concentration (0.2 mM). Volumes were 4.0 ± 1.1 μl/mg protein and 3.02 ± 0.99 μl/mg protein in control and

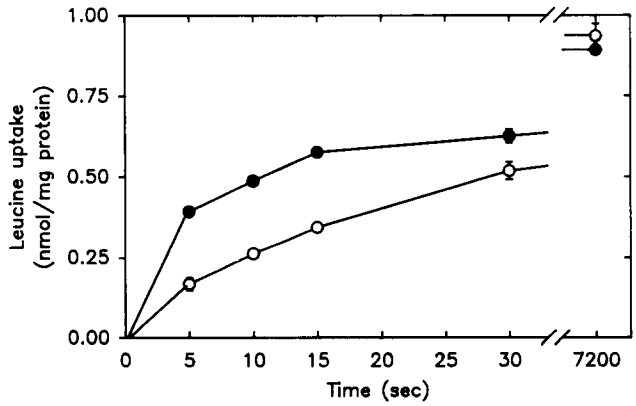

Fig. 5. Effect of PGO in the absence of a potassium gradient. Leucine uptake was determined as described in Fig. 1, except that vesicles were preloaded with 100 mM KCl, which is the same concentration present outside the vesicles in the radioactive cocktail. Closed symbols: control vesicles; open symbols: vesicles preincubated, at a protein concentration of 1.1 mg/ml, with 5 mM PGO. Data represent a typical experiment performed in triplicate. When absent, S.E. bars were smaller than the symbols used.

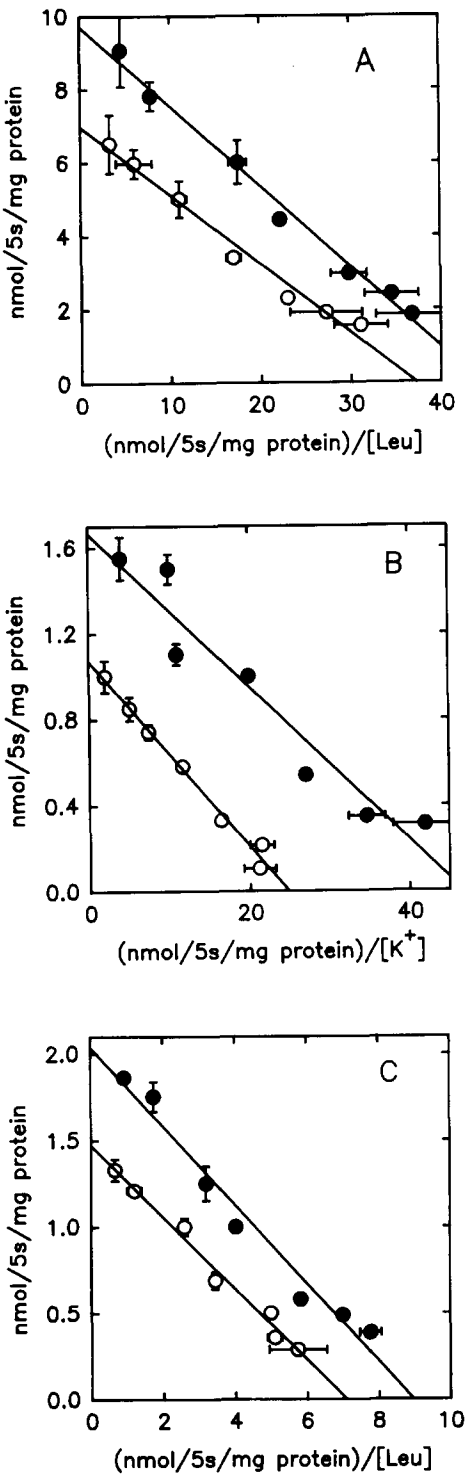

Fig. 6. Effect of PGO on the kinetics of leucine uptake. Eadie-Hofstee plots of the initial rates of leucine uptake determined at 5 s (the uptake is linear up to 12 s; see Ref. [8]) in the presence (Panels A and B) or in the absence (Panel C) of external KCl. Panels A and C: leucine uptake as a function of the external amino acid concentration; in Panel A the extravesicular K^+ was 150 mM. Panel B: leucine uptake as a function of the external potassium concentration. In Panel B leucine concentration was 0.1 mM. Closed symbols represent controls in the absence of PGO, open symbols vesicles, at a protein concentration of 5 mg/ml, preincubated for 15 min with 5 mM PGO.

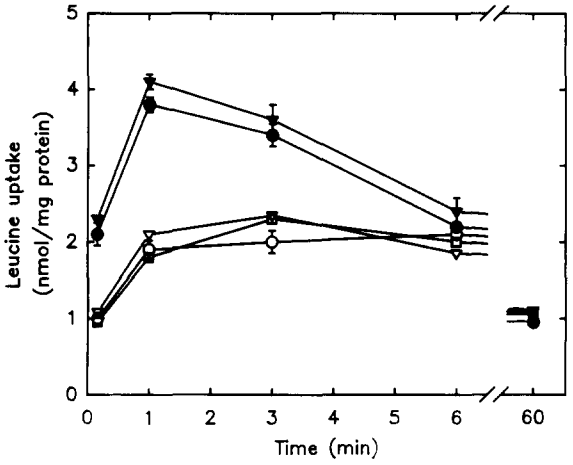

Fig. 7. Effect of protection of substrates binding sites on PGO inhibition of leucine uptake. Vesicles, at a protein concentration of 1.38 mg/ml, were preincubated 15 min with 5 mM PGO (□), 5 mM PGO + 50 mM leucine (▽), 0 mM PGO + 50 mM leucine (▼), 5 mM PGO + 500 mM KCl (○), 0 mM PGO + 500 mM leucine (●). Uptakes were determined as described in Fig. 1. Data represent a typical experiment performed in triplicate. When absent, S.E. bars were smaller than the symbols used.

treated vesicles, respectively (means ± S.E. of four different experiments).

In order to evaluate if the observed inhibition of amino acid uptake by PGO could be ascribed to the dissipation of the potassium gradient, leucine uptake in K^+ preequilibrated vesicles ($[K^+]_{out} = [K^+]_{in} = 100$ mM) was measured. As shown in Fig. 5, 5 mM PGO inhibited by 42% the initial rate of leucine uptake, without affecting the equilibrium value, confirming that the effect of the inhibitor was on the transporter.

Next, we examined the effect of PGO on the kinetics of leucine uptake as a function of external leucine or potassium concentration. The experiments were performed either with BBMV preincubated for 15 min in the presence of a fixed concentration of PGO or with untreated BBMV. The results are shown in Fig. 6, and the calculated kinetic parameters are given in Table 1. In all cases PGO inhibited leucine uptake by decreas-

Table 1
Effect of PGO on the kinetics of K^+/leucine cotransporter

Condition	V_{max} (nmol/5 s per mg protein)	K_m (mM)	
		Leu	K^+
A. KCl gradient			
Control	9.92 ± 0.21	0.23 ± 0.01	106 ± 19
5 mM PGO	7.17 ± 0.12 *	0.20 ± 0.01	117 ± 15
B. Without KCl			
Control	2.01 ± 0.10	0.25 ± 0.03	–
5 mM PGO	1.45 ± 0.09 *	0.22 ± 0.01	–

Parameters were calculated from the data reported in Fig. 6, by fitting the experimental results to the Michaelis-Menten curve. * $P < 0.05$ (Student's t-test), for comparison between control and treated vesicles.

ing the V_{max} value, without affecting the K_m. This was clearly evident also in the absence of K^+ (Fig. 6, panel C), i.e., when only the binary complex carrier-leucine is working [8].

To determine whether the two substrates were able to overcome the inhibition by PGO, preincubations were performed with substrates and inhibitor simultaneously present. Neither the presence of leucine nor that of potassium was able to affect the inhibitory potency of PGO (Fig. 7). This behaviour resembles that of an uncompetitive inhibitor suggesting that its interaction with the cotransporter was outside the binding site of both leucine and K^+.

4. Discussion

The present work represents the first study on chemical modification of a lepidopteran K^+-dependent amino acid cotransporter. Leucine transport across BBMV prepared from *P. cynthia* larvae was inhibited in a time- and dose-dependent manner by PGO, a specific reagent for arginine residues. At the IC_{50} value (5.1 mM), the half-time of inactivation ($t_{1/2}$) for leucine transport during the fast phase was 1.42 min. This value is lower than that determined under similar experimental conditions for glucose transport in BBMV from rat kidney ($t_{1/2}$ of 53 s at 50 mM PGO; see Ref. [20]. At the same PGO concentration, in our case $t_{1/2}$ value would be 9 s). Only the anion exchanger of the erythrocytes is characterized by a similar half-time of inactivation [25].

Vesicle integrity was preserved during PGO treatment and the inhibitory effect was observed also when a same potassium concentration was present at the two sides of the vesicle membrane, suggesting that inhibition was not related to the dissipation of the potassium gradient, which could have caused secondary inhibition by decreasing the driving force (Fig. 5).

The inhibition of leucine transport by PGO was not linked to changes in the affinity of the carrier for leucine or potassium, but was related to a decrease in the maximal velocity of the translocation process for either the ternary complex or the leucine-only form (Fig. 6). This suggests that PGO inhibition was not at the K^+-binding site. Moreover, since the inhibition was not removed by preincubating the vesicles with an excess of leucine (Fig. 7), arginine residues are not involved in the amino acid binding site.

In most of the enzymes studied, PGO reacts only with the arginine residues located at the catalytic site [26]. This high reactivity is the result of a lower pK_a of the guanidinium group, which in turn depends on a restricted microenvironment [27]. Although PGO has been reported to have a high selectivity for the arginyl group at pH 7.4 [28], its use has been criticized mainly because of the presence of side-reactions with thiol and primary amino groups, albeit with several orders of magnitude lower efficacy [29]. The presence of highly reactive thiol groups in the K^+/amino acid cotransporter seems to be excluded by the lack of any effect of 5 mM dithiothreitol on the K^+-dependent leucine transport (data not shown), a concentration that has been demonstrated to rapidly inactivate ($t_{1/2} < 10$ min) the mammalian Na^+/glucose cotransporter [30].

In Hepes-buffered solution, the rate of PGO-induced inactivation did not follow a simple first-order mechanism, which would be consistent with the presence of two or more populations of arginyl groups being modified. However, the substitution of borate buffer for Hepes buffer did alter the inactivation pattern of leucine transport, giving rise to a mono-exponential decay of the transport activity (Fig. 3). Since borate is known to maintain a strict one-to-one stoichiometry of addition of PGO to arginines [31], in non-borate buffer the inactivation of the carrier is the result of the addition of a first PGO molecule, followed by addition of a second PGO molecule to the arginine-PGO complex, which is prevented by the presence of borate.

In vertebrates, several symporters [19,20] and antiporters [21], including mitochondrial carriers [32], have been shown to contain essential arginine residues. However, the function of the guanidinium group in these transporters is not clear. A major role in the correct orientation of the protein in the membrane is certainly plausible. For the renal organic anion exchanger it has been tentatively proposed [21] that an arginine residue may serve to funnel the substrate to or away from the binding site involved in the translocation process. Waiting for more definite experiments on purified transporters, it is now clear that arginine residues play a crucial role in the translocation step of mammalian transporters for inorganic and/or organic substrates as well as of insect K^+-driven amino acid cotransporter. The search for structural differences between these two classes of transporters represents a challenging task in order to explain the lack of cation specificity of insect amino acid transporters.

5. Acknowledgement

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6. References

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