KAAT1 and CAATCH1 are amino acid transporters cloned from the intestine of the lepidoptera Manduca sexta.\(^1,2\) They are members of the SLC6/NSS family, which groups membrane proteins that use Na\(^+\), K\(^+\) and Cl\(^-\) gradients for the coupled transport of amines and amino acids. The report of the atomic-resolution x-ray crystal structure of the eubacterium Aquifex aeolicus leucine transporter (AaLeuT)\(^3\) has contributed significantly to understanding of the structure-function relationship in NSS proteins. Transport by AaLeuT is Cl\(^-\) independent, whereas many neurotransmitter:sodium symporters, such as serotonin transporter (SERT), GABA transporter (GAT1), dopamine transporter and norepinephrine transporter, among others, are strongly Cl\(^-\) dependent.\(^4\)

A single Cl\(^-\) ion is found bound to one of the extracellular loops, EL2 in AaLeuT. The Cl\(^-\) is 20 Å away from the Na and leucine binding sites, and thus it is unclear whether this Cl\(^-\) binding site is physiologically important. The nature of the association of Cl\(^-\) ions with these proteins during transport remains to be resolved. The Cl\(^-\) binding site of two members of the family, the serotonin transporter SERT\(^*\) and the GABA transporter GAT1,\(^5\) has been recently modeled on the basis of their functional properties and by structural homology to AaLeuT. The analyses have highlighted the role of a serine residue, that in the Cl\(^-\)-independent AaLeuT corresponds to Glu 290, and of an asparagine (Asn 286) that also contributes to the coordination of Na\(^+\) in the Na1 binding site of AaLeuT. KAAT1 and CAATCH1 are able to transport different amino acids depending on the cotransported cation (Na\(^+\) or K\(^+\)) but their Cl\(^-\) dependence is not completely defined yet. With the aim to clarify the role exerted by chloride in SLC6/NSS transporters, their transport activities resulted weakly Cl\(^-\)-dependent compared to GAT1.

Chloride’s Role in the Prototype Transporter of the SLC6 Family, GAT1

Na\(^+\) activated cotransporters move Na\(^+\) down its electrochemical gradient to drive the secondary active transport of ions, solutes, nutrients and neurotransmitters. This large group of cotransporters is composed of different gene families, which may show additional ionic requirements, as in many members of the SLC6 (solute carrier 6) or NSS (neurotransmitter sodium symporter) that are also dependent on chloride. Members of this family are involved in the uptake of neurotransmitters from the synaptic cleft and are targets for psychostimulants, anti-depressants and other drugs.\(^6\)

The prototype of this family is rGAT1, the GABA transporter cloned from rat brain in 1990, and its chloride dependence has been extensively studied.\(^7\) Expression of GAT1 in Xenopus laevis oocytes has confirmed the stoichiometry of 1GABA:2Na\(^+\):1Cl\(^-\), already proposed by studies performed in native membranes;\(^8-12\) nevertheless, the role played by Cl\(^-\) in the transport cycle is not yet completely understood. In fact, from a thermodynamic point of view, coupling with chloride, in addition to Na\(^+\), does not seem to confer a significant energetic advantage, since the chloride equilibrium potential in most cells is close to the resting membrane potential and therefore little free energy is supplied by the chloride electrochemical gradient. However electrophysiological analysis of GABA-induced currents in giant membrane patches\(^10\) and uptake measurements in liposomes\(^5\) show a potent inhibitory effect of high internal chloride, supporting chloride coupling in the transport process.

Another study, based on charge/flux ratio determinations, confirmed previous indications of a role of extracellular chloride in increasing the affinity for Na\(^+\), and it also suggested that chloride does not contribute to the net charge transferred during a complete transport cycle, because entry of a chloride ion during inward translocation of substrates would be balanced by export of another chloride ion during the second part of the cycle, in which the transporter returns to the outward facing conformation.\(^13,14\) Furthermore, it has also been reported that at very negative potentials the dependence of GABA-induced currents on extracellular chloride is not absolute,\(^9,10\) raising the possibility that under these conditions another anion, such as hydroxyl, may take the role of chloride.\(^15\)
External chloride affects in a similar way the pre-steady-state currents exhibited by many cotransporters in the absence of substrate: lowering the external chloride concentration produces a shift of the pre-steady-state currents toward more negative membrane potentials similar to that induced by Na⁺. The fact that two oppositely charged ions produce qualitatively similar effects indicates different mechanisms of action: whereas the effects of Na⁺ may be explained with a combined Boltzmann-Hill approach, the observed Cl⁻ action may be interpreted as favoring the transporter affinity for Na⁺. Finally, the “uncoupled” or “leak” transmembrane current, seen in many transporters in this family in absence of organic substrate has never been reported as chloride-dependent. This is also true for a unique current that is activated by GABA but not coupled with its transport, which has been recently described.

The Putative Chloride-Binding Site from the AaLeuT Structure

The report of the crystal structure of the eubacterium Aquifex aeolicus leucine transporter, AaLeuT, a member of the SLC6 family, has provided the framework for new studies. The AaLeuT structure revealed 12 transmembrane helices that create enclosed binding sites for two Na⁺ ions and one leucine molecule approximately midway across the lipid bilayer. Interestingly, in contrast to most other members of the family, the transport activity of AaLeuT is chloride-independent. The single chloride ion found in the crystal structure, probably does not have a physiological relevance for the transport activity. In fact it was positioned close to the extracellular loop 2, in a non-conserved region, far (20 Å) from the sodium and substrate binding site.

Honig and Kanner groups recently published two reports about the chloride role in the mammalian serotonin transporter SERT and in GAT1 respectively. Using different approaches they identified residues crucial for the chloride binding and clarified the role performed by this anion. Honig and his group combined calculations of pKₐ, homology modeling and site-directed mutagenesis. First, they searched for acidic residues in buried regions in the AaLeuT structure that could take the place of the negative charge contributed by the bound chloride, in chloride dependent members of the family. They identified the charged residue Glu290 as the best candidate. Indeed this residue is not present in the chloride-dependent transporters and corresponds to Ser372 in SERT (see Table 1). Based on the structure of AaLeuT, they created a homology model of the chloride-binding site in SERT with a chloride ion placed at the position of the E290 AaLeuT carboxylate carbon. The residues involved in the model of SERT are Y121, S336, N368 and S372 (corresponding to Y47, T254, N368 and E290 in AaLeuT) (Table 1). These residues are located in the TM2, 6 and 7, and they form a coordination shell for chloride very close to the Na1 binding site. Replacing S372 in SERT with Glu and Asp residues, which are present at the corresponding positions of chloride-independent prokaryotic transporters such as AaLeuT, almost completely abolished the chloride-dependence of SERT. The same result was observed for N368, when it was converted into an Asp, a residue present in several insect and prokaryotic transporters (Table 1). Thus, the introduction of a carboxylate at either S372 or N368 led to chloride-independent forms of SERT.

Kanner and his colleagues started from the crystal structures of two bacterial chloride channels, in which the chloride ions are coordinated by main-chain NH groups and by side-chain hydroxys from serine and tyrosine residues. They therefore focused their attention on serine, threonine and tyrosine residues located in the transmembrane domains that were conserved in the SLC6 transporters, but not in those where the transport is chloride-independent, such as AaLeuT. By this approach they pinpointed Ser331 in GAT1, located in TM7 near the Na1 binding site (see Table 1).

Negatively charged amino acid substitutions at Ser331 caused a very low transport activity in the mutants S331D and S331E, approximately 6% and 12% of WT GAT1, probably because this region of the protein is well conserved in the family. They observed that the residual GABA transport by the mutant was chloride-independent. Similar results were obtained in two other neurotransmitter transporters of the SLC6 family, the dopamine transporter DAT and in another GABA transporter, GAT4, when a glutamate residue was introduced at the position corresponding to residue 331 of GAT1. The reciprocal mutation in AaLeuT (E290S) rendered substrate binding chloride dependent, although transport activity could not be measured.

Lowering the internal pH of the liposomes could enhance the low transport activity of GAT1 S331E/D, whereas no effects were seen in WT GAT1. The interpretation given by the authors is that, after the cytoplasmic unloading of the substrates, the rate of return to the outward facing conformation is slowed down by the presence of a negative charge in (or near) position 331 of GAT1. In the WT transporter the charge carried by chloride is released, whereas it is fixed in S331E/D, but reducing the internal pH may neutralize it. Thus, both the Honig and the Kanner groups identified the same chloride-binding site in some transporters in this family. The close proximity of the chloride binding site to the Na1 binding site provides a good explanation for the tight coupling of the inward movement of the two ions and suggests that the chloride ion could be useful in the stabilization of the sodium binding.

KAAT1 and CAATCH1 Chloride Dependence

Mentioning a brief passage from Amara 2007 on this subject, “these new data also underscore the utility of studies on transporter family members from more distantly related organisms that may have evolved unique ionic dependencies to cope with the diverse environments they encounter”, we may introduce our work on the role of chloride in two invertebrate transporters, KAAT1 and CAATCH1, cloned from the gut of Manduca sexta lepidopteran larvae. These cotransporters have an unusual cation dependence being activated by Na⁺, K⁺ and Li⁺, and they are members of the SLC6 family as they show 35–45% identity with some vertebrate neurotransmitter transporters of this family. KAAT1 and CAATCH1 are very similar to

| Table 1 | Residues involved in the putative chloride binding sites |
|---------|---------------------------------------------------------|
| KAAT1/CAATCH1 | Cl⁻-dependent | Cl⁻-independent |
| Y93 | Y121 | Y86 | Y47 |
| Q302 | Q332 | Q291 | Q250 |
| S306 | S336 | S295 | T254 |
| D338 | N368 | N327 | N286 |
| T339 | C369 | S328 | E287 |
| S342 | S372 | S331 | E290 |
KAAT1 and CAATCH1 Are Weakly Cl⁻-Dependent

Figure 1 shows the chloride-dependence of amino acid uptake for KAAT1, CAATCH1 and GAT1, expressed in *Xenopus laevis* oocytes. The uptakes of leucine, proline and threonine (A), of proline and threonine (B) and of GABA (C) were measured in the presence of 100 mM NaCl or Nagluconate. Uptake was measured after 1 hr incubation in a solution having the following composition: 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES/NaOH, pH 8, supplemented with 500 KBq/ml of the tritiated amino acid. In the absence of chloride, gluconate salts were used. Data are presented as a percentage of the uptake measured in the presence of NaCl and are mean ± SE of 30 oocytes obtained from 3 different batches.

![Figure 1](image.png)

**Figure 1.** Uptake of 100 μM leucine, proline, threonine and GABA induced by KAAT1, CAATCH1 and GAT1, expressed in *Xenopus laevis* oocytes. White bars represent the uptake measured in the presence of NaCl 100 mM, gray bars the uptake in the presence of 100 mM Nagluconate. Uptake was measured after 1 hr incubation in a solution having the following composition: 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES/NaOH, pH 8, supplemented with 500 KBq/ml of the tritiated amino acid. In the absence of chloride, gluconate salts were used. Data are presented as a percentage of the uptake measured in the presence of NaCl and are mean ± SE of 30 oocytes obtained from 3 different batches.

In contrast to the majority of the other members of the family, which are rather strict both in ionic and substrate selectivity, KAAT1 and CAATCH1 are able to exploit the K⁺ electrochemical gradient (in addition to the Na⁺ gradient) to drive the active transport of the different neutral amino acids. Furthermore, the potency order with which various neutral amino acids are transported depends on the driver ion. These characteristics open the possibility that the chloride-dependence of the two transporters might change according to the driver ion and/or to the organic substrate.

Other residues in the vicinity of Ser342 might also play a role in determining the chloride sensitivity. In the predicted chloride-binding site of GAT1 there is another interesting residue (Ser328), which corresponds to Glu287 in AaLeuT (See Table 1). The mutations S328D/E in GAT1 resulted in a substantially chloride-independent transport, suggesting a role for other negative residues located nearby. Indeed, an aspartate residue is present in position 338 in both KAAT1 and CAATCH1 (See Table 1). This amino acid has been reported to play a role in conferring K⁺ sensitivity to these transporters, but its possible role in chloride sensitivity has not been yet investigated.
varied from 20 to 40% according to the amino acid being transported.

To better understand the chloride effect, leucine uptake was measured as a function of external chloride concentration (Fig. 2). The same Figure shows for comparison the Cl\(^-\) activation curve of GABA transport by GAT1 (inset). The Cl\(^-\) dependence of GAT1 was fitted with a hyperbolic curve with a \(K_{50}^{Cl}\) value of 9 ± 1.7 mM. By contrast, chloride concentrations below 30 mM did not affect leucine uptake in KAAT1 expressing oocytes. Only at higher concentrations a modest increase of leucine uptake was observed, in agreement with the data reported in Figure 1. Even if these data do not allow us to calculate \(K_{50}^{Cl}\) values for chloride activation, they indicate a very low apparent affinity of this anion for KAAT1.

The uptake data were complemented with electrophysiological experiments comparing the transport-associated current in normal and reduced external chloride concentration in controlled membrane potential conditions. To avoid circumstances that may have caused previous contradictory results, a reduced concentration of Cl\(^-\) was left (25 mM) to minimize the junctional potential effects, and the Ca\(^{2+}\) concentration was elevated to 10 mM to compensate for the Ca\(^{2+}\)-chelating properties of gluconate. Furthermore saturating concentrations of amino acids were used. Figure 3 shows the voltage-dependence of the transport currents elicited by the indicated amino acids in presence of Na\(^+\) in KAAT1 and CAATCH1. In all cases, with the exception of leucine in KAAT1 (but notice the relatively large errors), the chloride reduction caused a decrease of the transport current ranging between the 23 and 44%, in good agreement with the uptake data.

Voltage clamp experiments allowed us to investigate the behaviour of these two transporters when the driver ion was potassium. Indeed, without control of the membrane potential the high external K\(^+\) concentration necessary to energize transport is counteracted by the K\(^+\)-induced depolarization of the membrane, resulting in a very low electrochemical gradient. The results in potassium are shown in Figure 4, in which the various panels correspond to the analogous panels of Figure 3. For all amino acids tested, and for both transporters, the results do not differ from those in Na\(^+\).

On the whole, the electrophysiological observations and the uptake results indicate that both

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**Figure 3.** Transport-associated currents (currents in presence of amino acid minus current in its absence) elicited by threonine 3 mM (A and D), leucine 1 mM (B) and proline 3 mM (C and E) in presence of Na\(^+\) in KAAT1 (top row) and CAATCH1 (bottom row). Squares are currents in 100 mM chloride, circles are currents in 25 mM chloride, 75 mM gluconate. Data represents means ± S.E. of the mean from 3–8 oocytes from at least two batches.

**Figure 4.** Transport-associated currents (currents in presence of amino acid minus current in its absence) elicited by threonine 3 mM (A and C), leucine 3 mM (B) and proline 3 mM (D) in presence of K\(^+\) in KAAT1 (top row) and CAATCH1 (bottom row). Squares are currents in 100 mM chloride, circles are currents in 25 mM chloride, 75 mM gluconate. Data represents means ± S.E. of the mean from 5–9 oocytes from at least three batches.
transporters are moderately chloride-dependent. The similar behaviour of KAAT1 and CAATCH1 is in agreement with their identical sequence in the putative Cl⁻ binding site (Table 1) and suggests that the previous observations indicating differences between the two transporters in this respect were probably affected by uncontrolled artefacts. Alternatively, the small effects observed in the absence of chloride were considered not relevant when compared with the strict Cl⁻ dependence in other transporters.

**Conclusion**

In conclusion, the two insect members of the SLC6 family we studied are both weakly Cl⁻-dependent, and this dependence is little affected by the driver cation and/or amino acid transported. The comparative analysis of the putative residues involved in Cl⁻-binding suggests that the intrinsic negative charge found in the Cl⁻-independent transporter ALeuT (namely E290 and E287) could be partially supplied by aspartate 338 in KAAT1 and CAATCH1. This non-conserved residue plays a role in the cation selectivity of these transporters;25 but it might also supply the negative charge that renders these transporters able to work in the absence of chloride. If this hypothesis will be confirmed by experiments showing an increased Cl⁻-dependence in mutantized transporters lacking this negative charge, the transport cycle model proposed4,5 would assume an even more general validity in the SLC6 family.

The phytogamous lepidopteran larvae are characterized by a very low NaCl concentration in the intestinal lumen and in the haemolymph (where on the contrary K⁺ concentration is unusually high); low NaCl concentration in the intestinal lumen and in the haemolymph even more generally in the SLC6 family. Cl⁻-binding suggests that the intrinsic negative charge found in the Cl⁻-dependent transporters;25 but it might also supply the negative charge that renders this transporters able to work in the absence of chloride. If this hypothesis will be confirmed by experiments showing an increased Cl⁻-dependence in mutantized transporters lacking this negative charge, the transport cycle model proposed4,5 would assume an even more general validity in the SLC6 family.

The phytophagous lepidopteran larvae are characterized by a very low NaCl concentration in the intestinal lumen and in the haemolymph (where on the contrary K⁺ concentration is unusually high); thus the biophysical features of KAAT1 and CAATCH1 may have evolved from a particular ionic environment.29 However, it should be noted that the apparent affinity of KAAT1 for sodium is very high, but it is low for chloride. Therefore, it is difficult to assess a physiological meaning to the weak Cl⁻ dependence of these transporters. A conceivable alternative to be explored is that OH⁻ and not Cl⁻ are the physiologically anions, considered the very high pH and low Cl⁻ concentrations present in the midgut of these larvae.30-32

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