Structural domains involved in substrate selectivity in two neutral amino acid transporters

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The ability of the two highly homologous Na$_{+}$/K$_{+}$/Cl$_{+}$/H$_{11001}$/H$_{11002}$/Cl$_{2}$/H$_{11002}$-dependent neutral amino acid transporters KAAT1 and CAATCH1, cloned from the midgut epithelium of the larva Manduca sexta, to transport different amino acids depends on the cotransported ion, on pH, and on the membrane voltage. Different organic substrates give rise to transport-associated currents with their own characteristics, which are notably distinct between the two proteins. Differences in amplitude, kinetics, and voltage dependence of the transport-associated currents have been observed, as well as different substrate selectivity patterns measured by radioactive amino acid uptake assays. These diversities represent useful tools to investigate the structural determinants involved in the substrate selectivity. To identify these regions, we built four chimeric proteins between the two transporters. These proteins, heterologously expressed in Xenopus laevis oocytes, were analyzed by two-electrode voltage clamp and uptake measurements. Initially, we exchanged the first three domains, obtaining the chimeras C3K9 and K3C9 (where numbers indicate the transmembrane domains and letters represent the original proteins), which showed electrophysiological and [$^{3}$H]amino acid uptake characteristics resembling those of KAAT1 and CAATCH1, respectively. Subsequent substitution of the last four domains in C3K9 and K3C9 gave the proteins C3K5C4 and K3C5K4, which showed the same behavior as KAAT1 and CAATCH1 in electrophysiological and transport determinations. These results suggest that in KAAT1 and CAATCH1, only the central transmembrane domains (from 4 to 8) of the protein are responsible for substrate selectivity.

Comparing sequences and functions of related proteins can give some insights on the physiological role of particular domains. In the present study, we analyzed two amino acid transporters cloned from the Manduca sexta larva, the tobacco hornworm, which is a lepidopteran of great agroecological importance and is one of the best-studied insects as a result of the ease in rearing it on an artificial diet. The intestine of this larva has been the source for the cloning of the two transporters used in the present work: K$^{+}$-coupled amino acid transporter 1 (KAAT1) (6) and cation-amino acid transporter/channel 1 (CAATCH1) (9). The two proteins, of 634 and 633 amino acids, respectively, are very similar to each other (90% amino acid identity), and their 35–45% identity with the members of the Na$^{+}$/Cl$^{−}$/H$^{+}$/Cl$_{2}$/H$_{11002}$/Cl$^{2}$/H$_{11002}$-dependent neurotransmitter transporters allows them to be included in this important superfamily (6, 9). Accordingly, their hydropathicity profiles suggest the presence of 12 transmembrane domains with cytosolic carboxy and amino termini. The two transporters share the peculiar property of being able to utilize the K$^{+}$/Cl$^{−}$ gradient to energize uptake, a characteristic related to the high-K$^{+}$/low-Na$^{+}$/low-pH$_{11001}$/low-pH$_{11002}$ condition of the larva intestine (11, 12). However, although differing in only 63 amino acids, they exhibit specific electrophysiological properties and different organic substrate selectivities. The ionic dependence of the transport-associated current is different in the two transporters: at variance with KAAT1, CAATCH1 appears to be Cl$^{−}$ independent (9); in the presence of Na$^{+}$, KAAT1 is able to transport threonine, proline, methionine, and leucine (in this order of preference), whereas CAATCH1 transports only proline and threonine (in this order), with leucine and methionine having, instead, the effect of blocking the leak current (9). In the presence of K$^{+}$, KAAT1 transports leucine better than it does methionine and threonine but does not transport proline anymore; in the presence of K$^{+}$, the transport of proline by CAATCH1 is strongly impaired and becomes less efficient than that of threonine, whereas, surprisingly, the transport of methionine becomes possible (9).

Clearly, these distinct functional differences, together with the high degree of identity between KAAT1 and CAATCH1, represent a favorable situation in which to attempt the construction of chimeric transporters between these two proteins, with the aim being the identification of structural regions involved in substrate recognition.

To develop a strategy for the construction of the chimeras, we considered two aspects. First, we took into account the schematizations emerging from the literature suggesting that the transporter sequences may be subdivided in regions with specialized, though nonexclusive, functional roles (10, 17, 20): 1) the amino-terminal region, up to the fourth putative transmembrane segment, which should include ion dependence and the permeation pathway, and 2) the central and carboxy-terminal regions, responsible for the recognition of organic substrates and possibly involved in the interaction with inhibitors. Second, we considered the restriction maps of the two proteins: because of the high homology of the two sequences, it was easy to find common restriction sites in appropriate positions to construct chimeric proteins without the necessity.
and Functional Genomics, University of Florida, Gainesville, FL) were subcloned between the NcoI and HindIII sites of the high-expression vector pAMV-PA, kindly provided by Dr. C. Labarca (Division of Biology, California Institute of Technology, Pasadena, CA) (19). The NcoI restriction site was created by point mutation (Stratagene QuickChange) for both cDNAs in correspondence to the ATG codon; this site was then reverted to the original nucleotide sequence after cloning to reinsert the original amino acid sequence and to destroy the introduced NcoI site.

Using two restriction sites that were in the same position in CAATCH1 and KAAT1, NcoI (499) and PmlI (1276), and the NsiI site in the multiple cloning site of pAMV-PA, we constructed four chimeric proteins having [with reference to the putative 12-transmembrane domain topology suggested by hydrophobicity profiles (6, 9)] the first three transmembrane domains of KAAT1 and the last nine transmembrane domains of CAATCH1 (K3C9), or vice versa (C3K9), or the first three and last four transmembrane domains of KAAT1 and the central five transmembrane domains of CAATCH1 (K3C5K4), or vice versa, as shown in Fig. 2. The correct construction of the cDNAs was controlled by restriction analysis and complete sequencing (MWG-Biotech).

cRNA preparation and Xenopus laevis oocyte expression. The experimental procedure was described in detail elsewhere (7). Briefly, the cDNAs encoding the original cotransporters and the chimeras were linearized with NotI and cRNAs were synthesized in vitro in the presence of Cap analog and 200 units of T7 RNA polymerase. All enzymes were supplied by Promega Italia (Milan, Italy).

Xenopus laevis frogs were anesthetized in a 0.10% (wt/vol) solution of MS222 (tricaine methansulfonate) in tap water; portions of ovary were removed through a small incision on the abdomen that was subsequently sutured, after which the animal was returned to water. The oocytes were treated with 1 mg/ml collagenase (type IA; Sigma) in Ca2+- and Cl−-free ND-96 for at least 1 h at 18°C. Healthy-looking stages V and VI oocytes were collected and injected with 12.5 ng of the selected cRNA in 50 nl of water, using a manual microinjection system (Drummond). The oocytes were incubated at 18°C for 3–4 days in NDE solution (ND-96 solution; 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES supplemented with 50 μg/ml gentamicin and 2.5 mM Na-pyruvate at pH 7.6), before electrophysiological studies.

Electrophysiology and data analysis. A two-microelectrode voltage clamp was used to perform electrophysiological experiments (GeneClamp; Axon Instruments, Union City, CA). The holding po-

Fig. 1. Evolutionary tree constructed from the amino acid sequences as explained in text. The subfamilies of the γ-aminobutyric acid (GABA), monoamine, amino acid, “orphan,” and bacterial transporters are indicated. The 2 shaded sequences, K3C9- and K3C5K4-coupled amino acid transporter 1 (KAAT1) and cation-amino acid transporter/channel 1 (CAATCH1), were used to prepare the constructs shown in Fig. 2.

of introducing point mutations. Obviously, it is very likely that different parts of the sequence might contribute to the substrate specificity; it has been suggested that the three extracellular loops IV, V, and VI might form a “pocket” to which the substrate may bind (27). Studies performed on other proteins of the family suggest a role for the central domains of the protein, a region where KAAT1 and CAATCH1 differ for only 21 amino acids, with 15 conservative and 4 semiconservative substitutions; considering their position in the hypothetical tertiary structure and their chemical characteristics, only about half of them appear to be adequate to play a role in substrate recognition: G188S, G190N, E200Q, D286Q, W288A, A298S, A313P, L332W, S370A, and L416F (where the first letter is the amino acid in KAAT1 and the last is the amino acid in CAATCH1). Therefore, we decided to focus our attention from the external loop II to transmembrane domain VIII, where differences are present in significant number. As a consequence of these considerations, we constructed chimeras between KAAT1 and CAATCH1 in which three or four transmembrane domains were substituted in either the amino- or the carboxy-terminal regions and constructed others in which the five central domains of the protein were exchanged. The chimeras were expressed in Xenopus oocytes, and their functional characteristics were studied with electrophysiological measurements and radioactive uptake assays.

MATERIALS AND METHODS

Evolutionary tree. Representative sequences of the various subfamilies to which the Na+- and Cl−-dependent neurotransmitter transporters belong were employed to carry out a molecular phylogenetic reconstruction of the family (Fig. 1). This analysis shows the likeness among the considered sequences, extending previous observations (15, 18) to the recently cloned insect transporters. The sequences of the evolutionary tree are available in the protein database Swiss-Prot (http://www.expasy.org/sprot/). The sequences were aligned by using the program Clustal X 1.81 (13) and were then run using the software package TREECON (26) with the use of the neighbor-joining algorithm (23) for the reconstruction, based on a distance matrix and the distance correction method, as described previously (14).

Chimeric cDNA construction. The cDNAs coding for KAAT1 and CAATCH1 (generous gift of Dr. B. R. Stevens, Dept. of Physiology
tential was kept at −60 mV, and the typical protocol consisted of 200-ms voltage pulses spanning the range from −140 to +20 mV in 20-mV steps. Four pulses were averaged at each potential; signals were filtered at 1 kHz and sampled at 2 kHz. Experimental protocols, data acquisition, and analyses were done using the pCLAMP 8 software (Axon Instruments).

Solutions. The external control solution had the following composition (in mM): 98 NaCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES; in the other solutions, NaCl was replaced by KCl or tetramethylammonium chloride (TMA-Cl). The pH was adjusted to 7.6 by adding the corresponding hydroxide for each alkali ion and TMAOH for TMA⁺ solution. When chloride was replaced, gluconate salts were used. Amino acids (leucine, threonine, proline, and methionine; 500 μM) were added to induce transport-associated currents. Solutions were superfused by gravity onto the oocyte by a pipette tip placed very close (1–2 mm) to the cell.

Uptake experiments. Amino acid uptake was measured 3 days after injection. Groups of 10–12 oocytes were incubated in 100 μl of uptake solution (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES-Tris, pH 8) with 0.5 mM [³H]leucine, [³H]threonine, (37 MBq/ml; Amersham Pharmacia Biotech) for 60 min, and then oocytes were washed in ice-cold wash solution (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES-Tris, pH 8) and dissolved in 250 μl of 10% SDS for liquid scintillation counting. In K⁺-free experiments, NaCl was replaced by 150 mM KCl. The higher osmolality did not influence uptake (not shown). Experiment in which the expression level of KAAT1 or CAATCH1 was less than five times above background were discarded. The data reported in the figures show KAAT1-, CAATCH1-, and chimera-mediated transport representing the difference in the mean uptake measured in cRNA-injected oocytes and the mean uptake measured in noninjected oocytes.

RESULTS

Functional expression of chimeras. All constructs were able to transport proline, a good substrate for both KAAT1 and CAATCH1, in Na⁺, and to elicit transport-associated currents, although variability in the potency of the response among batches and among constructs was frequently observed. For this reason, we chose to focus our attention on aspects that could allow unequivocal distinction between KAAT1-like and CAATCH1-like behaviors, such as relative changes in selectivity order or reversal in current direction, rather than on amplitude differences in mean current or uptake.

Leak current and Cl⁻ dependence. A conspicuous “leak” or “uncoupled” current has been observed in both KAAT1 and CAATCH1-expressing oocytes (1, 2, 9, 21), and it has been attributed to a channel-like ionic flow through the transporter when the organic substrate is absent. The presence of this characteristic has been incorporated into the name of CAATCH1: “‘CH’ stands for ‘channel.’ We compared the properties of the leak current in the two transporters to verify its usefulness in distinguishing the behavior of our chimeras. The leak current was operatively determined as the difference between current level in the presence of Na⁺ or K⁺ and current level in TMA⁺ solution (1, 2). The results, shown in Fig. 3A, suggest that the amplitude and selectivity of the uncoupled current in the two transporters do not differ sufficiently to represent a useful criterion for discrimination.

In contrast to KAAT1, CAATCH1 also has been reported to lack Cl⁻ dependence (9), and this might have been another difference for distinguishing the chimeras. The lack of Cl⁻ dependence in CAATCH1, however, has been observed only in the presence of proline (9), whereas the Cl⁻ dependence in KAAT1 has been observed in the presence of leucine (6). We therefore tested the effects of Cl⁻ in KAAT1 by using proline and obtained the results illustrated in Fig. 3B, showing that KAAT1 is Cl⁻ independent when the organic substrate is proline and ruling out the utility of this test for distinguishing the chimeras.

Transport-associated current in the presence of either Na⁺ or K⁺. The ability of KAAT1 and CAATCH1 to handle different neutral amino acids in the presence of either Na⁺ or K⁺ was initially checked by measuring the transport-associated currents at a holding potential of −60 mV and applying Na⁺ or K⁺ solutions containing 500 μM of one of the four amino acids (leucine, threonine, methionine, or proline). The results in Fig. 4 show that the published observations (9) are confirmed and extended. In particular, in CAATCH1 leucine does not give rise to inward transport-associated currents in either Na⁺ or K⁺, whereas methionine is able to generate currents when the transporter is bathed in K⁺ but not in Na⁺; conversely, in CAATCH1, proline is able to elicit currents only in the presence of Na⁺. In KAAT1, all four amino acids support inward currents in the presence of Na⁺, whereas in the presence of K⁺, proline does not.

On the basis of these results, it is clear that the behavior of the two transporters differs most evidently in the lack of leucine- and methionine-induced inward currents by CAATCH1 when the driver ion is Na⁺ and, somehow less strongly, in the reversal in the order of preference between leucine and threonine when the carrier ion is K⁺. Furthermore, in the presence of Na⁺, proline is a good substrate for both transporters (21), whereas leucine is not transported by CAATCH1 (see Fig. 9) and therefore seems the best choice for discriminating the functional properties of the chimeras in uptake assays.

Fig. 3. A: leak current obtained by subtracting current in tetramethylammonium (TMA⁺) solution (I_TMA) from current in ion solutions (I_ion) containing Na⁺ (light shaded bars) or K⁺ (dark shaded bars) as the main cation in oocytes expressing KAAT1 (n = 22) and CAATCH1 (n = 25) and in noninjected oocytes (n = 3). Data represent means ± SE from 5 batches of oocytes. B: proline-induced (500 μM) transport currents (I_tr) in the presence (open symbols) or absence (filled symbols) of chloride (gluconate substitution). Data represent means ± SE from 4 oocytes from the same batch. V_m, membrane potential.
Effects of organic substrates on chimeras. To evaluate the behavior of the different chimeras, and on the basis of the indications of Fig. 4, we then measured the transport currents generated by exposure of the chimeras to leucine and methionine in presence of Na\(^+\) and to leucine and threonine in the presence of K\(^+\). Typical traces in Na\(^+\) are shown in Fig. 5, top: in the C3K9 and C3K5C4 chimeras, both leucine and methionine elicited inward transient currents, i.e., a response qualitatively similar to the transport-associated current they induced in KAAT1 in the same conditions. In the K3C5K4 and K3C9 chimeras, the current was in the outward direction, consistent with the block of the leak current reported for CAATCH1 in the presence of Na\(^+\) (9).

Figure 5, bottom, shows instead the behavior of the various constructs when exposed to leucine or threonine in the presence of K\(^+\). The relative amplitudes of the currents elicited by the two amino acids reverted from a KAAT1-like prevalence of leucine in C3K9 and C3K5C4 to a threonine dominance, similar to CAATCH1 in K3C5K4 and K3C9. Clearly, the behavior of the different chimeras in these tests suggests that the organic substrate specificity is in all cases strongly influenced by the parental sequence present in the five central transmembrane domains.

Current-voltage relationships. To obtain a more complete description of the behavior of the chimeras, we extended this analysis to a larger voltage range and to include other amino acids as well. Figures 6 and 7 show the current-voltage relationships derived from experiments in the two ionic conditions with different amino acids. The results in the presence of Na\(^+\) (Fig. 6) confirm that only those constructs having the KAAT1 central domains are able to generate an inward current when leucine is added, whereas in CAATCH1 and CAATCH1-like chimeras, leucine consistently causes an outwardly directed difference current for potentials negative to \(-40 \text{ mV}\). Furthermore, proline and threonine remain the preferred amino acids in all constructs, with the potency order gradually changing from a slight prevalence of threonine at all voltages in KAAT1 to a prevalence of proline at low voltages (in C3K9) and to a more marked prevalence of proline at all voltages in all other constructs and in CAATCH1. When the main ion present is K\(^+\) (Fig. 7), leucine is the amino acid producing the largest currents at all potentials in the constructs having the KAAT1-derived central region, although threonine also generates similar currents in C3K5C4. However, threonine definitely becomes the most potent substrate in the constructs having the CAATCH1-derived central region, which also shows some ability of proline to induce inward current at negative potentials.

Methionine block of proline-induced currents. An interesting observation in the behavior of CAATCH1 is the ability of a low concentration of methionine to block the large transport current elicited by proline (9). We attempted to determine whether this characteristic could be used as a further test for distinguishing KAAT1-like behavior from CAATCH1-like behavior in the chimeras. The illustration in Figure 8 shows that in KAAT1, the action of methionine is not additive to that of proline but, similar to that in CAATCH1, is competitive: progressive additions of methionine to a fixed 0.5 mM proline stimulation caused a reduction of the inward current to the level reached by 0.5 mM methionine alone. Chimeras C3K9 and C3K5C4 behaved like KAAT1 in this experiment, and, conversely, K3C9 and K3C5K4 behaved like CAATCH1, showing a stronger block by methionine, as already reported (9). This experiment therefore represents another confirmation of the relevance of the central section of the transporters in determining the substrate specificity.

Uptake measurements. To corroborate the electrophysiologically derived data, we also measured the uptake of \(^{[3H]}\)leucine, \(^{[3H]}\)proline, and \(^{[3H]}\)threonine in oocytes expressing the wild-type and chimeric forms of KAAT1 and CAATCH1 in the presence of Na\(^+\) or K\(^+\). It is noteworthy that in the presence of 150 mM KCl, the oocyte membrane potential is close to zero, and consequently, the amino acid uptake mediated by KAAT1 and CAATCH1 is much lower than in the presence of NaCl. Figure 9A shows that in the presence of Na\(^+\), proline and threonine are good substrates for both transporters, whereas CAATCH1 does not mediate leucine uptake. All the tested chimeras mediate proline and threonine uptake, and only K3C5K4 and K3C9 do not transport leucine, reproducing the behavior of CAATCH1.
C3K5C4 and C3K9 show a transport pattern similar to that of KAAT1, though with reduced activity. Figure 9B shows that in the presence of K\(^+\), despite the limitations of the experimental conditions, the preferences of CAATCH1 for threonine and of KAAT1 for leucine are confirmed; in addition, KAAT1 is unable to transport proline. Chimeras behave in a way similar to the transporter providing the central region of the construct. In particular, C3K5C4 and C3K9 prefer leucine and have impaired proline transport, as does KAAT1; K3C5K4 and

Fig. 6. Current-voltage relationships from wild-type and chimeric transporters, as indicated, in the presence of Na\(^+\). Constructs with KAAT1-like behavior are shown at left, whereas constructs with CAATCH1-like behavior are shown at right. Data were obtained by subtracting the traces recorded in the absence from those recorded in the presence of the indicated substrate (500 μM). The data have been normalized to the value in proline at −140 mV for each oocyte before averaging. Values are means ± SE from 4–6 oocytes in each group.
K3C9 show a pattern of amino acid preference more similar to that of CAATCH1.

The structural composition of the chimeras and the transport activities shown in Fig. 9 therefore confirm that in both cotransporters, the amino-terminal region up to the first three transmembrane domains and the last four transmembrane domains are not involved in the amino acid discrimination. Consequently, the middle region is sufficient to confer substrate selectivity in both KAAT1 and CAATCH1.

DISCUSSION

The assessment of structure-function relationships in proteins is a natural goal of molecular physiology. The combination of electrophysiology, radiochemical assays, and molecular biology appears very promising for the purpose of identifying the structural domains involved in specific roles in the activity of membrane transport proteins. The construction of chimeras by using domains from transporters of the Na+/H+-dependent family has proved to be useful in delineating the structural determinants involved in the selectivity for catecholamines (4) or in the interaction with drugs (5, 10). The probability of obtaining functional chimeras is higher when the two parent proteins are similar, and indeed, the dopamine and norepinephrine transporters used in the above-cited works show high sequence identity (Fig. 1).

This also is true for proteins of relatively distant organisms, such as, for instance, the human (hSERT) and Manduca sexta serotonin transporters (MasSERT): MasSERT is comparatively less sensitive to cocaine than most members of the monoamine transporter subfamily, and this feature can be exploited to investigate drug selectivity. Studies on chimeras from these transporters suggest that transmembrane domains 1 and 2 affect substrate transport, possibly changing the transporter conformations (24). The two lepidopteran transporters used in our work, KAAT1 and CAATCH1, are 90% identical, yet they exhibit characteristic substrate selectivity sequences that may be exploited to identify the domains involved in substrate recognition.

Before the results obtained using our chimeras are discussed, it is perhaps worthwhile to expend a few words on the behavior of the wild-type transporters. KAAT1 and CAATCH1 are exceptional among ion-coupled cotransporters in being able to function using the K⁺ gradient in addition to the Na⁺ gradient (6, 9), and we know that this property is related to the specific ionic conditions of the larval intestine, which is rich in K⁺ and poor in Na⁺ (22). However, Na⁺ and K⁺ are not completely equivalent in both proteins: the current-voltage relationships...
are more strongly inwardly rectifying when the carrier ion is K\(^+\) than when it is Na\(^+\) (see Figs. 6 and 7; Ref. 1, 6). In KAAT1, the current-voltage curve in the presence of K\(^+\) is negatively shifted compared with that in Na\(^+\), and this corresponds to the more negative position of the intramembrane charge movement measured in the absence of organic substrate; this difference is likely to have a physiological meaning, considering the very negative value of the apical membrane potential (8).

The qualitative correspondence of the voltage dependence of pre-steady-state and transport-associated currents in both Na\(^+\) and K\(^+\) (1) is in agreement with a similar correlation observed in the rat GABA transporter rGAT1, and in that case, it has been explained with a kinetic model in which ionic interaction and charge translocation must precede the binding of the organic substrate. This might constitute the basis for the understanding of another interesting observation in KAAT1 and CAATCH1: namely, the fact that for each transporter, the substrate selectivity order changes depending on whether K\(^+\) or Na\(^+\) is the main bathing ion. Figures 6 and 7 show that whereas proline generates the largest currents in KAAT1 in the presence of Na\(^+\), this amino acid is the least effective in the presence of K\(^+\). Analogously, in CAATCH1, whereas proline is the preferred amino acid in the presence of Na\(^+\), the largest current in the presence of K\(^+\) is produced by threonine.

In our interpretation, the picture that emerges from these observations and considerations is one in which Na\(^+\) or K\(^+\) may first interact, each with its own voltage dependence characteristics, with the transporters, creating a structural moiety to which each substrate amino acid may bind and trigger the subsequent transport steps with its peculiar kinetics. In this view, for instance, the moiety created by the complex Na\(^+\)-KAAT1 may be highly favorable for the interaction with proline, whereas that originated by the K\(^+\)-KAAT1 complex might be much less so. In summary, the overall turnover rate of the transporters might depend on the kind of ternary complex formed by the 1) transporter, 2) ion(s), and 3) organic substrate.

**Leak current and its block.** The leak current properties in the absence of organic substrates appear to be so similar in KAAT1 and CAATCH1 that they cannot be exploited in structure-function studies performed in chimeric constructs between the two transporters. The Cl\(^-\) independence of the proline-induced current, as reported for CAATCH1 (9), is also present in KAAT1 (Fig. 3B), whereas the Cl\(^-\) dependence of leucine transport, as exhibited by KAAT1 (6), cannot be studied in CAATCH1. Again, the Cl\(^-\) dependence of the two transporters does not appear to be a useful test for discriminating purposes.

Although present in many cotransporters, leak currents are particularly conspicuous in CAATCH1 and KAAT1. We have speculated that this may be so to favor absorption of Na\(^+\) from the K\(^+\)-rich intestinal lumen (1). The reduction of the membrane current by an organic substrate, which may even be accompanied by transport (3, 21), would represent a slowing down of the turnover rate, instead of its increase. In other words, there may be a basal cycling rate of the transporter, in the absence of organic substrate, that may be either increased or decreased, depending on the kind of interacting amino acid and on the ionic conditions. The interaction of methionine and proline, first described for CAATCH1 (9) but also observed in the present study in KAAT1 (Fig. 8), is very interesting in this respect. In both transporters, proline is able to increase the basal turnover rate; methionine instead accelerates the turnover rate in KAAT1 (to a lesser degree compared with proline) but slows it in CAATCH1.

The current-voltage relationships shown in Figs. 6 and 7 under leucine block are rather intriguing: the apparently outward current induced by leucine in the presence of Na\(^+\) is slightly larger in the chimeras with the central CAATCH1 domains compared with CAATCH1; in the presence of K\(^+\), leucine may induce an inward current at potentials more positive than \(-80\) mV, with a positive shift in the zero current potential compared with Na\(^+\). However, these observations are affected by some uncertainties, because the leucine block of the leak current is not complete (Figs. 4 and 5), the voltage dependence of the leak current is shifted to more negative potentials in the presence of K\(^+\) anyway (1), and, furthermore, the contribution of the current carried by the oocyte endogenous channels (especially in K\(^+\)) is not known. Given these reservations, we prefer not to derive any deductions from these kinds of observations.

**Determinants of substrate selectivity.** Our results from the chimera studies show that the central transmembrane domains, from IV to VIII, strongly determine the selectivity order for the recognition of organic substrates (Figs. 5, 6, 7, and 9). The 63 different amino acids (10%) are variably distributed along the entire sequences of KAAT1 and CAATCH1: 8 (1.3%) of the different amino acids are in the amino-terminal region (from the beginning to the NcoI site), with 5 conservative substitutions; 21 (3.4%) are in the central region (between NcoI and the PmlI sites), with 15 conservative and 4 semiconservative changes; and finally, 34 (5.3%) are located in the carboxy-terminal region (from PmlI to the end), with 18 conservative and 6 semiconservative changes. The eight amino acids located in the first three transmembrane domains do not appear to play any role in substrate selectivity; this finding is not surprising, because the amino terminus is the most conserved region in these two proteins and also in the whole Na+/Cl\(^-\)-dependent transporter family, which suggests that this region is involved in general transport functions shared by all members of the family. Some quantitative differences in the degree of efficacy of the various amino acids (see Figs. 6 and 7) may indicate that the carboxy-terminal region, where the two transporters show a high variability, may nevertheless give only a very small contribution to the selectivity characteristics. Therefore, the remaining 21 different residues located between transmembrane domains IV and VIII appear to be the best candidates for a functional role in the structures responsible for substrate recognition.

It has already been suggested (25) that the region including domains IV, V, and VI may form a substrate binding pocket in the related transporter rGAT1; recently, the fourth extracellular loop has also been involved in substrate interaction (27). Working on KAAT1, our group has also shown that aspartate 338, presumably located in the transmembrane domain VII, is implicated in cation selectivity and in the coupling between cation and amino acid fluxes (16). The present data seem to extend the role of this region in the functioning of K\(^+\)-coupled lepidopteran transporters. In the region encompassed by transmembrane domains IV to VIII, KAAT1 and CAATCH1 differ for only 21 residues, which will possibly be mutated individ-
ually and in combinations to further restrict the definition of the structural determinants of the substrate specificity of these transporters.

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REFERENCES


