The Aromatic and Charge Pairs of the Thin Extracellular Gate of the γ-Aminobutyric Acid Transporter GAT-1 Are Differently Impacted by Mutation*  

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Oshrat Dayan, Assaf Ben-Yona, and Baruch I. Kanner

From the Department of Biochemistry and Molecular Biology, Institute for Medical Research Israel-Canada, Hebrew University Hadassah Medical School, Jerusalem 91120, Israel

Background: The extracellular gate in GAT-1 contains aromatic and charge pairs.

Results: The impact of mutation of the pairs is different.

Conclusion: The aromatic pair ensures a high apparent GABA affinity.

Significance: This provides new insights in the molecular mechanism of neurotransmitter transport.

GAT-1 is a sodium- and chloride-coupled GABA transporter and a member of the neurotransmitter:sodium:symporters, which are crucial for synaptic transmission. The structure of bacterial homologue LeuT shows a thin extracellular gate consisting of a charge and an aromatic pair. Here we addressed the question of whether mutation of the aromatic and charge pair residues of GAT-1 has similar consequences. In contrast to charge pair mutants, significant radioactive GABA transport was retained by mutants of the aromatic pair residue Phe-294. Moreover, the magnitude of maximal transport currents induced by GABA by these mutants was comparable with those by wild type GAT-1. However, the apparent affinity of the non-conserved mutants for GABA was reduced up to 20-fold relative to wild type. The voltage dependence of the sodium-dependent transient currents of the Phe-294 mutants was similar to that of the wild type. On the other hand, the conserved charge pair mutant D451E exhibited a right-shifted voltage dependence, indicating an increased apparent affinity for sodium. In further contrast to D451E, whereas the extracellular aqueous accessibility of an endogenous cysteine residue to a membrane-impermeant sulphydryl reagent was increased relative to wild type, this was not the case for the aromatic pair mutants. Our data indicate that, in contrast to the charge pair, the aromatic pair is not essential for gating. Instead they are compatible with the idea that they serve to diminish dissociation of the substrate from the binding pocket.

The neurotransmitter:sodium:symporters (NSS) remove their substrates from the synaptic cleft. Thereby, these transporters ensure that the concentrations of the neurotransmitters in the synapse are sufficiently low so that the postsynaptic receptors can detect transmitter molecules newly released by exocytosis. With exception of the transporters for glutamate, the transporters for other neurotransmitters, such as GABA, serotonin, dopamine, norepinephrine, and glycine, are sodium- and chloride-dependent and belong to the NSS family (reviewed in Refs 1 and 2). These eukaryotic NSS transporters couple the flux of neurotransmitters not only to that of sodium but also to that of chloride, and in the case of GABA transporter GAT-1, the stoichiometry of the coupled electrogenic transport is 2Na⁺:1Cl⁻:GABA (3–6). Therefore, in addition to radioactive GABA uptake, the transport can also be monitored by measuring GABA-induced steady-state currents (5, 6). Although the precise binding order is not fully established, it is clear that influx is initiated by the binding of at least one sodium ion, followed by chloride and GABA (Fig. 1A). In the absence of substrate, sodium binding can be indirectly monitored by measuring capacitative transient currents, which reflect a charge-moving sodium-dependent conformational change. These sodium-dependent transient currents appear to be due to the transition of the negatively charged transporter from the inward facing to the outward facing conformation (Fig. 1A, step 5) and subsequent stabilization of the latter conformation by the binding of sodium (Fig. 1A, step 1) (7). The addition of GABA enables the full transport cycle (Fig. 1A), and the transient currents are thereby converted into GABA-dependent steady-state currents (5).

High resolution structures of the bacterial NSS homologue LeuT in different conformations have been described (8, 9). LeuT appears to be an excellent model for the NSS neurotransmitter transporters (10–13). LeuT consists of 12 TMs with TMs 1–5 related to TMs 6–10 by a pseudo-2-fold axis in the membrane plane, and its binding pocket is located at the interface of these two domains (8). In the outward occluded conformation, the binding pocket is separated from the cytoplasm by ~20 Å of ordered protein. In addition to this “thick” cytoplasmic gate, the structure contains a “thin” extracellular gate, which is composed by conserved charge pair and aromatic pairs (8). The charge pair, formed by Arg-30 from TM1 and Asp–404 from TM10, is located just “above” Tyr-108 from TM3 and Phe-253 from TM6, which constitute the aromatic pair (Fig. 1B). The hydroxyl group of the side chain of Tyr-108 interacts...
directly with one of the carboxy-oxygens of the amino acid substrate (8) (Fig. 1B), and therefore even the most conservative substitution mutants of the GAT-1 equivalent Tyr-140 are devoid of any transport activity (14). The comparison of LeuT structures crystallized in various conformations suggests that during gating, the cation-π interaction between Arg-30 and Asp-451 of LeuT. Even the most conserved substitution mutants, R69K and D451E, are defective in transport but exhibit sodium-dependent transient currents (7, 15). The voltage dependence of the transient currents by these mutants reflects an increased apparent affinity for sodium (7, 15). These mutant transporters are predominantly outward facing (7), as expected from a defect of their extracellular gate. The defective transport by these mutants indicates that the precise interaction of these charge pair residues is a prerequisite for the transition from the outward facing transporter (in T°) to the loaded outward facing transporter (in T°) (14). The comparison of LeuT structures crystallized in various conformations suggests that during gating, the cation-π interaction between Arg-30 and Asp-451 of LeuT. Even the most conserved substitution mutants, R69K and D451E, are defective in transport but exhibit sodium-dependent transient currents (7, 15). The voltage dependence of the transient currents by these mutants reflects an increased apparent affinity for sodium (7, 15). These mutant transporters are predominantly outward facing (7), as expected from a defect of their extracellular gate. The defective transport by these mutants indicates that the precise interaction of these charge pair residues is a prerequisite for the transition from the outward facing transporter to the inward facing transporter. Here we address the question of whether the aromatic pair, Tyr-140 and Phe-294 in GAT-1, fulfills a role in gating similar to that of the charge pair.

**EXPERIMENTAL PROCEDURES**

**Generation and Subcloning of Mutants**—Mutations were made by site-directed mutagenesis of GAT-1 in the vector pBluescript SK− (Stratagene) using single-stranded uracil-containing DNA as described previously (17, 18). Briefly, the GAT-1 containing plasmid was used to transform *Escherichia coli* CJ236 (Fig. 1B) is preserved because they rotate together (9).

The GAT-1 residues Arg-69 and Asp-451 are equivalent to the charge pair residues Arg-30 and Asp-404 of LeuT. Even the most conserved substitution mutants, R69K and D451E, are defective in transport but exhibit sodium-dependent transient currents (7, 15). The voltage dependence of the transient currents by these mutants reflects an increased apparent affinity for sodium (7, 15). These mutant transporters are predominantly outward facing (7), as expected from a defect of their extracellular gate. The defective transport by these mutants indicates that the precise interaction of these charge pair residues is a prerequisite for the transition from the outward facing transporter to the inward facing transporter. Here we address the question of whether the aromatic pair, Tyr-140 and Phe-294 in GAT-1, fulfills a role in gating similar to that of the charge pair.

**RESULTS**

**GABA Transport by Phe-294 Mutants**—In the HeLa cell expression system, radioactive GABA transport by F294Y was similar to that by GAT-1-WT (Fig. 2). Most of the other Phe-294 mutants showed markedly reduced but significant [3H]GABA transport, and only in the case of F294G was transport nondetectable (Fig. 2). Nevertheless, the impact of muta-
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The GABA concentration used for radioactive transport is generally far below the $K_m$ and was 23 nM (87.1 Ci/mMol), as compared with the $K_m$ value of 1–2 μM for GAT-1-WT (26). The reason is that large amounts of radioactive substrate are required to obtain a signal in the presence of saturating concentrations of unlabeled substrate, in particular for mutants with an increased $K_m$. This limitation is absent when using an assay without radioactivity, namely GABA-induced transport currents. Using this assay, it is trivial to determine whether the reduced radioactive transport by the Phe-294 mutants was due to an increased $K_m$ and/or a reduced $V_{\text{max}}$. In NaCl-containing media, saturating concentrations of GABA elicited robust inward rectifying currents in Xenopus oocytes expressing GAT-1-WT (Fig. 3A). The same was not only true for F294Y, but also for the other Phe-294 mutants, as exemplified here for F294C (Fig. 3A). The amplitude of the sodium-dependent steady-state currents induced by saturating GABA concentrations, $I_{\text{max}}$, by the Phe-294 mutants was comparable with that by GAT-1-WT (Fig. 3 legend). The voltage dependence of the transport currents by the Phe-294 mutants was similar, but not identical, to that by GAT-1-WT (Fig. 3B). Remarkably, examination of the dependence of $I_{\text{max}}$ on the GABA concentration showed that most of the Phe-294 mutants exhibited a decrease of the apparent affinity for GABA of ~15-fold (Fig. 4). Even F294G exhibited GABA-induced currents, but its apparent affinity for GABA was lower than any of the other Phe-294 mutants; its $K_m$ was ~20-fold higher than that of GAT-1-WT (Fig. 4). In con-
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FIGURE 5. Sodium-dependent transient by GAT-1-WT and Phe-294 mutants. A, transient currents of oocytes expressing GAT-1 WT, the indicated Phe-294 mutants, and D451E were measured using the voltage jump protocol described in Fig. 3. All traces shown are from representative oocytes, which are typical for at least three oocytes. The transient currents are defined as the currents in ND96 minus those in ND96 plus 10 μm tiagabine. The dashed lines indicate zero current. B, fit of the charge movements to a Boltzmann distribution as a function of the potential. The charge movements of oocytes expressing WT-GAT-1, F294C, F294Y, F294I, and F294A in 100 mm sodium and D451E also in 30 mm sodium were plotted as a function of the voltage. Charge movements were normalized to $Q_{max}$ and fitted using the Boltzmann distribution nonlinear curve fit function in Origin 6.1 (OriginLab Corporation). Wherever error bars are not visible, the error was smaller than the size of the symbols. The $Q_{max}$ values of WT, F294Y, F294C, F294A, and F294I were 14.3 ± 1.4, 16.1 ± 1.6, 16.4 ± 3.2, 15.1 ± 1.8, 11.4 ± 1.5, and 7.5 ± 1.5 nC, respectively. Note that at 100 mm sodium, the charge movements of D451E do not reach saturation, and in this case the charge movements were normalized to those at +60 mV. The data points are averaged from at least three oocytes for each transporter. C, transient currents by oocytes expressing F294Y/D451E and F294C/D451E mutants are shown. The scale bars refer to A and C.

Contrast, the F294Y mutant, which had similar radioactive transport as GAT-1-WT (Fig. 2), also had a similar apparent affinity for GABA using substrate-induced currents as a readout of transport (Fig. 4). The results with the Phe-294 mutants were in marked contrast with those of the charge pair mutants. The latter did not exhibit measurable GABA-induced currents, as exemplified with D451E (Fig. 3A).

Sodium-dependent Transient Currents by Phe-294 Mutants—All of the Phe-294 mutants tested exhibited sodium-dependent transient currents, as shown here for F294Y, F294C, F294A, and F294I (Fig. 5A). These currents are capacitative, because transient currents of the same magnitude but opposite direction were seen in the “off” phase when jumping back to the holding potential. The voltage dependence of the transient currents by the F294Y and F294C mutants was similar to that of GAT-1-WT (Fig. 5, A and B). The values of $V_{1/2}$, the voltage at which the charge movements are half-completed, for GAT-1-WT, F294Y, and F294C in the presence of 100 mm sodium were $-42.4 ± 1.1$, $-44.5 ± 2.2$, and $-29.4 ± 0.7$ mV, respectively. This is in contrast to the voltage dependence observed with the transient currents by D451E, where in the “on” phase only outward transient currents were seen (Fig. 5A). Even at potentials as positive as +60 mV, the charge movements by D451E were not yet saturated (Fig. 5A), and therefore it was not possible to determine $V_{1/2}$. Only when the sodium concentration was reduced from 100 to 30 mm, was saturation reached with a $V_{1/2}$ value of $-36.8 ± 1.2$ mV for D451E at 30 mm, which is close to the values for GAT-1-WT, F294Y, and F294C obtained in the presence of 100 mm sodium (Fig. 5B). Thus the apparent affinity of D451E for sodium is roughly 3.3-fold higher than that of GAT-1-WT, F294Y, and F294C. Therefore, as opposed to GAT-1-WT and the Phe-294 mutants, apparently all of the D451E transporters are already in the sodium-bound state at $-25$ mV, which is the holding potential in these experiments. The bound sodium can be released by jumps to more positive potentials, resulting in the outward transient currents. In contrast to D451E, the voltage dependence of the transient currents by F294A and F294I is left-shifted, with $V_{1/2}$ values of $-61.7 ± 1.3$ and $-63.5 ± 0.8$ mV, respectively, indicating a lowered apparent affinity for sodium. The analysis of the charge movements also allows for the calculation of $z$ for $\delta$, where $z$ is the charge on the particle moving, and $\delta$ is the fraction of the membrane field through which the charge moves. The values for $z$ were $1.31, 1.40, 1.41, 1.23,$ and $1.57$ for GAT-1-WT, F294C, F294Y, F294I, and F294A, respectively. Except for F294A, these values are in reasonable agreement with data on GAT-1-WT from the literature (5). For D451E at 30 mm Na, the value of $z$ was $1.37$. The voltage dependence of the transient currents by the F294Y/D451E and the F294C/D451E double mutants was similar to that of D451E alone (Fig. 5C), indicating that the phenotype of D451E is dominant over that of F294Y and F294C.

Transient Currents by Tyr-140 Mutants—None of the Tyr-140 mutants tested exhibited $[^{3}H]$GABA uptake, and the same is true for the GABA-induced transport currents (data not shown).
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FIGURE 6. Transient currents by GAT-1-WT and Tyr-140 mutants. A, transient currents of oocytes expressing GAT-1 WT and the indicated Tyr-140 mutants were measured using the voltage jump protocol described in Fig. 3. All traces shown are from the same oocytes, which are typical for at least three oocytes. Transient currents are defined as the currents in ND96 minus those in ChCl96. The dashed lines indicate zero current. B, fit of the charge movements to a Boltzmann distribution as a function of potential. The charge movements of oocytes expressing WT-GAT-1 and Tyr-140 mutants in 100 mM sodium were plotted as a function of the voltage. Charge movements were normalized to \( Q_{\text{max}} \) and were fit with the Boltzmann equation as described in Fig. 5. Wherever error bars are not visible, the error was smaller than the size of the symbols. In this series of experiments, the \( Q_{\text{max}} \) values of WT, Y140F, Y140A, and Y140C were 35.3 ± 4.0, 16.9 ± 3.3, 20.1 ± 2.3, and 26 ± 1.8 nC, respectively. The data points are averaged from at least three oocytes for each mutant.

Inhibition of the Transient Currents by MTSET—The reactivity of the endogenous cysteine, Cys-74, located in TM1 to the membrane-impermeant positively charged sulfhydryl reagent MTSET can be used as a readout of the extent to which the transporter is outward facing. As noted previously, this cysteine residue is relatively unreactive (19, 20). Preincubation of oocytes expressing GAT-1-WT with the relatively high concentration of 5 mM of the sulfhydryl reagent was much more pronounced inhibition by MTSET was observed, relative to GAT-1-WT (7) (Fig. 7). This indicates that at \(-25 \text{ mV} \) in the presence of 100 mM Na\(^+\), the proportion of outward facing D451E transporters is significantly higher than that in GAT-1-WT. As shown previously, the sodium-dependent transient currents by D451E/C74A were not sensitive to the sulfhydryl reagent (Fig. 7), indicating that the inhibition of the transient currents of D451E by MTSET is indeed due to the modification of Cys-74. In contrast, when the indicated Phe-294 and Tyr-140 mutants were analyzed by this assay, a diminished inhibition by MTSET was observed, relative to GAT-1-WT (Fig. 7).
DISCUSSION

The phenotype of mutations in the aromatic pair of the thin extracellular gate of GAT-1 is completely different from those in the charge pair: nonconservative mutants of Phe-294 exhibit low but significant radioactive transport at GABA concentrations far below saturation (Fig. 2), and the measurements of GABA-induced transport currents indicate that this is due to a markedly reduced apparent affinity for GABA (Fig. 4), rather than to the maximal transport rate. In contrast, no GABA-induced transport currents were observed even with the most conserved charge pair mutants such as D451E (Fig. 3) and R69K (15). As documented previously, mutants of Tyr-140, which is the “partner” of Phe-294, also did not exhibit transport (14), but this appears to be due to a direct interaction of the hydroxyl group of this amino acid residue with one of the carboxy-oxygen of the substrate (8). The specificity of the impact of mutations of Tyr-140 is illustrated by the fact that these mutants still were capable of mediating sodium-dependent transient currents (Fig. 6), which are a readout of the transition between the inward and outward facing conformation of the transporter and the stabilization of the latter by sodium binding (Fig. 1A, steps S and 1) (7). The voltage dependence of the transient currents by the Tyr-140 mutants indicates a similar or reduced apparent affinity for sodium compared with GAT-1-WT (Fig. 6, A and B), and the same is true for the Phe-294 mutants (Fig. 5, A and B). In contrast, the charge pair mutants exhibit an markedly increased apparent sodium affinity (Fig. 5, A and B) (7, 15). Apparently charge pair mutants have an increased probability to populate outward facing conformations, as inferred from aqueous accessibility of the endogenous Cys-74 to membrane-impermeant MTSET (Fig. 7) (7). Consistently, Phe-294 and Tyr-140 mutants with a lower apparent affinity for sodium, such as F294A and F294I (Fig. 5, A and B) and Y140C (Fig. 6, A and B), appear to be more inward facing because in the background of these mutations, Cys-74 is less sensitive to inhibition by MTSET (Fig. 7). At the present time, we do not have a good explanation for why some aromatic pair mutants have a decreased apparent sodium affinity and others do not. However, the important issue is that, unlike the charge pair mutants, none of the aromatic pair mutants have an increased probability to populate outward facing conformations.

How can we rationalize these observations? The fact that the transient currents are seen only in the most conserved charge pair mutations indicates these residues are basically irreplaceable for gating. The geometry of the charge pair apparently has to be very precise, because even with the conserved mutants D451E and R69K, no transport currents are observed (Fig. 3) (7, 15). The reason is presumably that only with aspartate and arginine residues at positions 451 and 69, respectively, there is an optimal interaction to seal the transporter from the extracellular medium (9), and this facilitates its opening toward the cytoplasm. On the other hand, even nonconserved aromatic pair mutations do not abolish the transient currents and do not result in an increased apparent affinity for sodium (Figs. 5 and 6). Moreover, even the nonconserved Phe-294 mutants are capable of transport with comparable maximal rates as GAT-1-WT (Figs. 3 and 4). This indicates that in the aromatic pair mutants, the gating is hardly affected, if at all. The drastically reduced apparent affinity of these mutants for GABA can be explained by the Michaelis-Menten formalism: $K_m$ equals $(k_{-1} + k_2)/k_1$, where $k_1$ and $k_{-1}$ represent rates of binding and unbinding of GABA, respectively, and $k_2$ represents the lumped rate constant of the steps in the transport cycle subsequent to the binding of GABA from the extracellular medium. The comparable $I_{\text{max}}$ values for the mutants indicate that the slow translocation steps ($k_2$) are apparently not markedly affected in the Phe-294 mutants. It is reasonable to assume that the 15–20-fold increase in $K_m$ by replacing Phe-294 to smaller residues is due to a pronounced increase in $k_{-1}$, the rate of unbinding of GABA toward the extracellular medium rather than an increased $k_1$. In the latter case, the $K_m$ values for the Phe-294 mutants should have decreased. In other words, it appears likely that the role of Phe-294 is to slow this rate of unbinding. Consistent with this idea is that when Phe-294 is replaced by the smallest residue, glycine, the impact on $K_m$ is the largest (Fig. 4). Our conclusions fit well with results of leucine binding to LeuT, showing that LeuT-F253A (corresponding to GAT-1-F294A) has a $K_m$ value that is more than 13-fold higher than that of LeuT-WT (27).

Because of the direct role of Tyr-140 in substrate binding, we cannot draw the same conclusion for this aromatic partner residue, although here the size of its side chain is also likely to prevent fast unbinding of the substrate. In any case, the direct
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Substrate liganding role of Tyr-140 also helps to stabilize GABA in the binding pocket of the transporter.

Our conclusions on the role of the aromatic pair in slowing down the "escape" of the substrate back to the extracellular medium can be further rationalized by considering the recent LeuT structures, reflecting different conformations of this homologue (8, 9). In the outward open conformation, the aromatic pair residues are at a distance of ~12 Å (see legend to Fig. 8) from each other, and for the charge pair, the distances are ~8 Å (Fig. 8, left panel). This is wide enough to let GABA, with a length of ~5 Å, enter the binding pocket. In the outward occluded structure of LeuT, the distances for each of the two pairs is similar, at ~5–6 Å (Fig. 8, middle panel). This would allow the substrate to unbind, but given the fact that these pairs are layered on top of each other, the rate most likely would probably be very slow. Replacement of one of the aromatic residues to a smaller one would result in an increase in distance between them from ~5 to ~8 Å. Therefore, escape of GABA would be easier, reflected in an increased value for \( k_{-1} \) and thereby of \( K_{m} \). In contrast to the charge pair, there is no direct interaction between the aromatic pair residues (9) (Fig. 8, right panel), even in the inward facing conformation. This could explain why there is only a minor impact of gating in the aromatic pair mutants (Figs. 5 and 6). The idea that the charge pair, but not the aromatic pair, controls gating is further supported by the observation that the former is the dominant determinant of the voltage dependence of the sodium-dependent transient currents (Fig. 5C).

In the transition from the outward open to the outward occluded conformation of LeuT, Arg-30 of the charge pair (equivalent to Arg-69 of GAT-1) is suggested "to ride on top of" Phe-253 (Phe-294 of GAT-1). This would maintain the potentially important cation-π interaction (9). However, our functional data with the nonconserved Phe-294 mutants show that this interaction is not important to achieve maximal transport rates, at least not in GAT-1 (Figs. 3 and 4). Such functional studies could also be important to determine whether the conserved amino acid residues participating in the intracellular gating network of the NSS transporters have different roles in the overall transport process (8, 9, 28, 29).

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