Molecular Basis for Substrate Discrimination by Glycine Transporters*

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Glycine is an inhibitory neurotransmitter in the spinal cord and brain stem, where it acts on strychnine-sensitive glycine receptors, and is also an excitatory neurotransmitter throughout the brain and spinal cord, where it acts on the N-methyl-D-aspartate family of receptors. There are two Na+/Cl−-dependent glycine transporters, GLYT1 and GLYT2, which control extracellular glycine concentrations and these transporters show differences in substrate selectivity and blocker sensitivity. A bacterial Na+-dependent leucine transporter (LeuTAA) has recently been crystallized and its structure determined. When the amino acid residues within the leucine binding site of LeuTAA are aligned with residues of the two glycine transporters there are a number of identical residues and also some key differences. In this report, we demonstrate that the LeuTAA structure represents a good working model of the Na+/Cl−-dependent neurotransmitter transporters and that differences in substrate selectivity can be attributed to a single difference of a glycine residue in transmembrane domain 6 of GLYT1 for a serine residue at the corresponding position of GLYT2.

The crystal structure of a bacterial Na+-dependent leucine transporter has recently been determined (10), and this protein shows remarkable amino acid sequence similarity with the mammalian family of Na+/Cl−-dependent neurotransmitter transporters, which include transporters for glycine, γ-aminobutyric acid, dopamine, serotonin, and norepinephrine (10, 11). The structure consists of 12 TM domains with TM1–5 and TM6–10 arranged in a pseudo-symmetric structure. The substrate binding site of LeuTAA is formed at the junction between the two regions and is composed of amino acid residues from TM1 and TM6. Both of these two transmembrane domains contain an unwound segment and many of the substrate contact sites are with the main chain atoms of these unwound segments (Fig. 1). Given the high degree of sequence similarity, Yamashita et al. (10) predicted that mammalian neurotransmitter transporters have very similar structures and that the neurotransmitter binding sites are located within analogous regions of their respective transporters. When the amino acid residues within the leucine binding site of LeuTAA are aligned with residues of the two glycine transporters there are a number of identical residues and also some key differences (Fig. 1). In this report we test the hypothesis that the LeuTAA structure represents a good working model of the Na+/Cl−-dependent glycine transporters and demonstrate that differences in substrate selectivity between GLYT1 and GLYT2 can be attributed to a single amino acid difference within the glycine recognition site. However, this difference cannot explain the differences in blocker selectivity.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were obtained from Sigma (Sydney, Australia) unless otherwise stated. NFPS was obtained from Tocris and dilutions were made from a stock solution of 740 mM in 50% Me2SO. ALX1393 was diluted from a stock solution of 100 mM in 30% Me2SO. ALX1393 was diluted from a stock solution of 740 μM in 30% Me2SO. The highest concentration of inhibitor, N[3-(4-fluorophenyl)-3-(4′-phenylphenoxo)propylsarcosine (NFPS)3 contains the sarcosine moiety and it is tempting to speculate that the specificity of NFPS for GLYT1 over GLYT2 may be due to the sarcosine moiety. However, in a previous study on the mechanism of action of NFPS on GLYT1 we were unable to demonstrate that NFPS competed with sarcosine or glycine in binding to GLYT1, which implied that the inhibitor and substrates do not share a common binding site (5).

The abbreviations used are: NFPS, N[3-(4-fluorophenyl)-3-(4′-phenylphenoxo)propylsarcosine; TM, transmembrane.

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Me$_2$SO applied to the cells was 0.04%, which did not cause a measurable effect.

Expression of Transporters in Xenopus laevis Oocytes and Electrophysiological Recordings—cDNAs encoding the human glycine transporter GLYT1b (12) and GLYT2a (13) were sub-cloned into the pTOV plasmid (14). Mutations in GLYT1b and GLYT2a were generated using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA), and all mutations were confirmed by DNA sequencing. The wild type and mutant transporters were linearized with SpeI and cRNA transcribed from the cDNA constructs with T7 RNA polymerase and capped with 5’-7-methyl guanosine using the mMessage mMachine kit (Ambion Inc., Austin, TX).

Oocytes were harvested from X. laevis, as described previously (15) with all procedures in accordance with the Australian National Health and Medical Research Council guidelines for the prevention of cruelty to animals. 50 nl of cRNA was injected into defolliculated, stage V oocytes, and incubated at 16 °C in standard frog ringer solution (ND96; 96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 5 mM HEPES, pH 7.55), supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 50 μg/ml gentamicin. 2–5 days later, current recordings were made at −60 mV using the two-electrode voltage clamp technique with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) interfaced with a MacLab 2e chart recorder (ADI Instruments, Sydney, Australia) using the Chart software (ADI Instruments). The expression of GLYT1 and GLYT1 mutants at the oocytes cell surface was checked using a cell surface protein biotinylation procedure (Pierce). Protein were labeled according to the manufacturer’s instructions and then oocytes were vortexed in a cell lysis buffer containing 1% Triton X-100, 0.1 M ing to the manufacture’s instructions and then oocytes were biotinylated proteins, the samples were analyzed by SDS-PAGE and Western blotting. A rabbit anti-GLYT1 antibody was obtained from David Pow (University of Newcastle, Australia) and was used at a dilution of 1: 1000. Goat anti-rabbit secondary antibodies tagged with alkaline phosphatase were used to detect GLYT1.

Charge-to-flux Ratios—Uptake of [3H]glycine (Amersham Biosciences, Sydney, Australia) was measured using voltage-clamp in oocytes expressing wild type and mutant transporters. Oocytes were voltage-clamped at −60 mV and 30 μM [3H]glycine was applied for 1 min and the transport current was recorded. This was followed by a 3-min washout and oocytes were removed from the bath and lysed in 50 mM NaOH, and scintillation counting was performed. Nonspecific [3H]glycine uptake was estimated by measuring [3H]glycine uptake by uninjected oocytes and was subtracted from uptake by oocytes expressing the glycine transporters. The amount of charge transfer associated with [3H]glycine uptake was determined by integrating the current measurement for the time of [3H]glycine application.

Data Analysis—The analysis of kinetic data were carried out using the Kaleidagraphe Software version 3.1. Current (I) as a function of substrate concentration ([S]) was fitted by least squares analysis to: $I/I_{\text{max}} = [S]/(E_{C_{50}} + [S])$ (Equation 1), where $I_{\text{max}}$ is the maximal current and $E_{C_{50}}$ is the concentration of glycine that generates a half-maximal current. Data for sarcosine-elicited currents were normalized to the maximal current generated by glycine for each cell.

RESULTS

In the crystal structure of the bacterial leucine transporter, LeuT$_{sa}$, the amino group of leucine is hydrogen-bonded to the main chain carboxyls of Phe-253 and Thr-254 and the hydroxyl group of the side chain of Ser-256. We have focused on the role of the corresponding residues in GLYT1 and GLYT2 in an attempt to explain why GLYT1 can transport the N-methyl derivative of glycine, sarcosine, while the GLYT2 subtype does not bind or transport sarcosine. An alignment of the amino acid sequences of GLYT1, GLYT2, and LeuT$_{sa}$ in the region proposed to bind substrate (10) is presented in Fig. 1. In GLYT1, the residue corresponding to the Ser-256 of LeuT$_{sa}$ that forms a hydrogen bond with the amino group of the substrate leucine is a Gly residue, whereas in GLYT2 the residue is a Ser. Furthermore, the neighboring residues are not conserved among the three transporters. These observations lead us to investigate whether the three differences highlighted in Fig. 1 are responsible for the differences in substrate and blocker selectivity between GLYT1 and GLYT2.

Applications of glycine to oocytes expressing either wild type GLYT1b or GLYT2a generate concentration-dependent inward currents (Fig. 2) that are due to the coupled transport of Na$^+$/Cl$^-$ and glycine (14, 16, 17). Sarcosine also generates concentration-dependent currents for GLYT1b but not GLYT2a (Fig. 2), which is indicative of sarcosine being a substrate for GLYT1b but not GLYT2a.

The Roles of GLYT1b-G305 and GLYT2a-S481—Application of glycine to oocytes expressing the GLYT2a mutant S481G
FIGURE 2. Glycine and sarcosine transport by wild type and mutant glycine transporters. A, representative current traces from oocytes expressing wild type and mutant glycine transporters perfused with 30 μM sarcosine (filled bars) or 30 μM glycine (open bars). For the GLYT2a-S481A mutant, 300 μM sarcosine and 300 μM glycine were used. B, concentration response curves for glycine (open circles) and sarcosine (filled circles) transport by wild type and mutant glycine transporters. Currents were normalized to the maximal current generated by glycine and fitted to Equation 1. Data represent mean values ± S.E. from n = 4–6 oocytes.
generate concentration-dependent inward currents that are of similar amplitude to wild type GLYT2a and wild type GLYT1b, but the EC_{50} for glycine transport currents is higher than both GLYT1b and GLYT2a (58 ± 6 μM for GLYT2a-S481G; 12.0 ± 1.1 μM and 20 ± 1 μM for GLYT2a and GLYT1b, respectively; Table 1). In contrast to wild type GLYT2a, sarcosine generates concentration dependent inward currents for the GLYT2a-S481G mutant. The maximal current generated by sarcosine is 97% (n = 7) of that generated by glycine and the EC_{50} for sarcosine induced currents for the mutant is 26.2 ± 1.3 μM (n = 7) (Fig. 2, Table 1). In comparison, sarcosine generates a maximal inward current in wild type GLYT1b of 87% ± 1% (n = 4) of that for glycine with an EC_{50} of 22 ± 1 μM (n = 4) (Fig. 2, Table 1). Application of glycine to the reverse mutant GLYT1-G305S generates inward currents, but these are considerably smaller in magnitude (<1 nA, n = 19, from four batches of oocytes) than the wild type transporters. It was not possible to measure accurately glycine or sarcosine concentration responses because of the small current amplitudes and the potential for confounding effects of high concentrations of glycine (>3 mM) on un.injected oocytes. The cell surface expression of GLYT1-G305S was confirmed by surface biotinylation followed by SDS-PAGE and Western blotting. The mutant protein was expressed at the cell surface but the levels were reduced to ~10% of wild type GLYT1, which could explain the substantially reduced transport currents.

The residue next to the serine/glycine difference in the GLYT1 also differs between the two transporters with a Cys residue in GLYT1b and an A in GLYT2a. In the LeuT_A crystal structure the corresponding residue points away from the bound leucine and as such is unlikely to directly interact with the substrate. However, the residues of transmembrane domain 6 immediately after this residue generate a break in the helix structure, which was suggested to play a role in creating a flexible part of the transporter that would be required for the transport process. Therefore, we investigated whether this residue difference alone, or together with the Ser/Gly difference, also played a role in determining differences in substrate transport.

Glycine generates inward currents in cells expressing the GLYT2a-A482C mutant, but in contrast to the S481G mutant, the substrate selectivity of this mutant is similar to the wild type GLYT2a. Applications of high doses of sarcosine (300 μM to 1 mM) did generate small currents (~1% of the maximal glycine current) with this mutant but it was not possible to reliably analyse these results. We also investigated whether sarcosine could bind to this transporter and inhibit glycine transport. However, doses of sarcosine up to 1000 μM have minimal effect on the amplitude of the current generated by 30 μM glycine (99 ± 2% of control glycine currents, n = 5 cells, data not shown), which suggests that sarcosine does not compete with glycine for occupancy of the transporter. The reverse mutation in GLYT1b, C306A, resulted in a wild type GLYT1b phenotype. Both glycine and sarcosine are substrates with EC_{50} values that are comparable with wild type GLYT1b (Fig. 2 and Table 1). These observations suggest that this residue difference between the two transporter subtypes does not play a role in determining the differences in substrate selectivity. Application of glycine to oocytes expressing the double mutant GLYT1b-G305S,C306A generated small inward currents (I_{max} 6.1 ± 1.2 nA, n = 6). Cell surface biotinylation assays demonstrated that the transporter was expressed at the surface but at substantially reduced levels compared with wild type, which could explain the small currents observed.

The EC_{50} for glycine is 140 ± 40 μM (n = 6), but in contrast to wild type GLYT1b, sarcosine at concentrations up to 1 mM did not generate inward currents. Thus, we conclude that as the GLYT1b-C306A mutant did not cause any substantial change in the GLYT1b substrate selectivity, then the differences in substrate selectivity observed for the double mutant can be attributable to the Gly to Ser change.

To investigate further the role of the amino acid side chain differences in GLYT1b and GLYT2a at this site we constructed GLYT1b-G305A and GLYT2a-S481A. Application of glycine and sarcosine to GLYT1b-G305A generates concentration-dependent inward currents with similar EC_{50} values to that of wild type GLYT1b (11 ± 2 and 14 ± 3, respectively, for the
mutant), with a maximal sarcosine-induced current of 110 ± 4% of the maximal glycine-induced current. Thus, either a Gly or an Ala residue are sufficient to allow sarcosine transport by GLYT1b. The cell surface expression levels of GLYT1b-G305A were ~60% of that for wild type GLYT1b. Glycine and sarcosine also generated inward transport currents when applied to oocytes expressing GLYT2a-S481A. The amplitude of the glycine transport currents were similar to that of wild type GLYT2a (84 ± 7 nA, n = 5), but the EC$_{50}$ values for glycine and also sarcosine were greater than the EC$_{50}$ for glycine transport by the wild type GLYT2a (1070 ± 80 μM for glycine (n = 5) and 590 ± 50 μM for sarcosine (n = 5) transport by GLYT2a-S481A compared with 12 ± 1 μM for glycine transport by GLYT2a). The maximal current generated by sarcosine was 70 ± 3% (n = 5) of that for glycine, which is comparable with that of wild type GLYT1b (87 ± 1%). Thus, for GLYT2a, sarcosine can be transported if an Ala or a Gly, but not a Ser residue, are present at this site.

**Substrate Selectivity of GLYT1b-Y302F and GLYT2a-F478Y**—The main chain carbonyl oxygen of Phe-253 of LeuT$_{A_5}$ forms a hydrogen bond with the amino group of leucine and at the corresponding residues in GLYT2a and GLYT1b there are Phe and Tyr residues, respectively. We investigated whether these differences may also contribute differences in substrate selectivity.

GLYT2a-F478Y and the reverse mutation in GLYT1b, Y302F were generated, but application of glycine or sarcosine to the GLYT2a-F478Y mutant did not generate any currents. Application of both glycine and sarcosine to the GLYT1b-Y302F mutant did generate transport currents that were considerably smaller than wild type GLYT1b. The maximal current generated by glycine was 6.9 ± 1.2 nA, with an EC$_{50}$ of 1.1 ± 0.2 μM and for sarcosine the maximal current was 77 ± 4% of that for glycine with an EC$_{50}$ of 1.8 ± 0.7 μM (Fig. 2 and Table 1). The cell surface expression of the GLYT1b-Y302F mutant was ~60% of that for wild type GLYT1b. These results demonstrate that the Y302F mutant is expressed at the cell surface and does alter the function of the transporter, but it does not appear to change the relative substrate selectivity.

**Blocker Sensitivities of Mutant Transporters**—NFPS is an irreversible blocker of GLYT1 and has no apparent effect on GLYT2a (5, 6). Co-application of 30 μM glycine with 1 μM NFPS to oocytes expressing GLYT1b, GLYT1b-G305A, or GLYT1b-C306A caused slow onset reductions in the current. After 40 s exposure to 1 μM NFPS and 30 μM glycine, the glycine evoked current decayed by 43 ± 3% for wild type GLYT1b, 31 ± 7% for GLYT1b-G305A, and 52 ± 3% for GLYT1b-C306A (Table 1). Co-application of 30 μM glycine with up to 10 μM NFPS to oocytes expressing GLYT2a, GLYT2a-S481G, and GLYT2a-A482C generated stable inward currents, which were of similar magnitude to glycine alone (data not shown).

ALX1393 is a GLYT2a selective transport blocker (18), and we also investigated whether the GLYT2a mutants showed any changes in sensitivity to this blocker. Co-application of 1 μM ALX1393 and 30 μM glycine to oocytes expressing the wild type GLYT2a caused a gradual decline in the transport current and after 40 s the current was reduced to 72 ± 5% of the current measured in the absence of ALX1393 (Table 1). ALX1393 caused similar reductions in the glycine transport currents for the GLYT2a mutants compared with the wild type GLYT2a (Table 1). Thus, the GLYT2a-S481G and GLYT2a-S481A mutations, which affect substrate selectivity, do not prevent ALX1393 from binding to the transporter. It is interesting to note that ALX1393 applied alone to oocytes expressing GLYT2a-S481G generated an outward current at ~60 mV (18 ± 3% of the amplitude of the 30 μM glycine current, Fig. 3), which is also apparent in the wild type transporter, but to a much lesser extent (7 ± 1% of the amplitude of the 30 μM glycine current, Fig. 3). Based on observations of the effects of transport blockers on other neurotransmitter transporters (19–21), the most likely explanation for these outward currents is that ALX1393 blocks a substrate-independent leak current and that the amplitude of the leak current is greater in the mutant than the wild type transporter.

Glycine transport by GLYT1 is coupled to the co-transport of 2 Na$^+$ ions and 1 Cl$^-$ ion (17), which generates a charge-to-flux ratio of 1. In contrast, transport by GLYT2a is coupled to the co-transport of 3 Na$^+$ ions and 1 Cl$^-$ generating a charge to flux ratio of 2. We measured the charge to flux ratios of GLYT1b, GLYT2a, and the GLYT2a mutant S481G to see if the change in substrate selectivity of the mutant also affected the ion coupling stoichiometry (Fig. 4). Oocytes expressing the transporters were voltage-clamped at ~60 mV and [H]glycine was applied for 60 s. The charge associated with transport was measured by integrating the current over the time of application and the amount of [H]glycine taken up was measured using scintillation counting. From these measures, the charge to flux ratio was determined. The experimentally determined values for GLYT2a and GLYT1b are 2.10 ± 0.04, respectively, which are consistent with the expected values. The charge-to-flux ratio for GLYT2a-S481G was 2.05 ± 0.10, which is similar to that of GLYT2a. This result suggests that, although the mutation has changed the substrate selectivity, the ion flux coupling ratio has not been altered. It was not possible to conduct similar experiments with the GLYT1b Y302F, G305S, G305A, and the G305S-G305A mutants because of the reduced transport current amplitudes.

**FIGURE 3.** ALX1393 blocks a leak current in wild type GLYT2a and GLYT2a-S481G. Representative current traces for ALX1393 block of glycine transport and block of a leak current in GLYT2a and GLYT2a-S481G. 1 μM ALX1393 (filled bar) blocks a leak current in both the wild type and the mutant, but the amplitude of this leak current is greater in the mutant. Applications of 30 μM glycine are indicated by the open bar.
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FIGURE 4. Charge-to-flux ratios for wild type GLYT1b, GLYT2a, and GLYT2a-S481G. Oocytes expressing the transporters were voltage clamped at -60 mV and 30 μM [3H]glycine was applied, and both the current and [3H]glycine uptake were measured. The total number of charges transferred across the membrane were calculated and divided by the number of glycine molecules taken up into the oocytes. Data represent mean ± S.E. (n = 6 oocytes for each transporter). See “Experimental Procedures” for more detail.

DISCUSSION

One of the key predictions from the crystal structure of the bacterial Na⁺/Cl⁻-dependent leucine transporter, LeuT<sub>AA</sub>, was that this protein is a good structural model for the mammalian Na⁺/Cl⁻-dependent neurotransmitter transporters (10). The LeuT<sub>AA</sub> structure is of sufficient resolution to visualize the substrate bound to the transporter, and it was possible to identify molecular contact sites between transporter and the substrate. From this information, and given the degree of amino acid sequence similarity between LeuT<sub>AA</sub> and mammalian neurotransmitter transporters, Yamashita et al. (10) were able to predict how the different transporters distinguish between different transporters.

In this study, we have directly tested the suggestion that LeuT<sub>AA</sub> is a good structural model for the neurotransmitter transporters and used this to better understand differences in substrate selectivity between the two glycine transporter subtypes, GLYT1 and GLYT2. Glycine is a substrate for both GLYT1 and GLYT2, while the N-methyl derivative of glycine, sarcosine, is a substrate for only GLYT1. In the LeuT<sub>AA</sub> structure the amino group of leucine is hydrogen-bonded to the side chain of Ser-256, and the residue corresponding to this residue in GLYT2 is Ser-481, whereas in GLYT1 the residue is Gly-305. In this study, we have demonstrated that switching the Ser-481 for a Gly residue in GLYT2 allows sarcosine transport with similar affinity and rate of transport as glycine. The reverse mutation in GLYT1b was not functional, but the mutant that contained the reverse mutation together with an additional mutation of the neighboring residue prevented sarcosine transport while still allowing glycine transport. Given that the single mutation in GLYT1b of C306A caused no change in substrate selectivity compared with wild type GLYT1, we conclude that it is the G305S mutation that is responsible for the change in the double mutant. Thus, these results suggest that the N-methyl group of sarcosine can be accommodated within the substrate binding site if a Gly residue is present at this position, but if the larger Ser residue is present then sarcosine cannot fit within the site.

These observations raise the question as to whether it is the size of the Ser side chain or the chemical nature of the side chain. To address this point we investigated the properties of alanine substitutions at these two residues. The amplitude of transport currents were reduced for the GLYT1b G305A mutant compared with wild type, but sarcosine does generate transport currents, which indicates that the larger sarcosine can still be accommodated within the substrate binding pocket. This suggests that it is the hydroxyl group that plays an important role in determining how the amino group of the substrate interacts within the site. It is interesting to note that the affinity of glycine for the GLYT1b-G305A mutant is similar to that of wild type GLYT1b. This suggests that the mutation does not cause any significant disruption to the way in glycine interacts with the transporter. The GLYT2a S481A mutant also transported sarcosine, which suggests that this larger substrate can still be accommodated within the site with the methyl side chain, but it cannot fit into the site with the methoxy group of Ser present. Thus, it appears that the hydroxyl group of Ser influences the way in which sarcosine can fit into the substrate binding pocket. The EC<sub>50</sub> values for substrate transport by this mutant were significantly larger than for wild type GLYT2a, which suggests that substitution of the Ser residue for an Ala residue may alter the way in glycine interacts with the binding site. Thus, the Ser for Gly difference between GLYT2a and GLYT1b influences whether sarcosine is substrate or not, but in addition the difference is also likely to influence the way in which both glycine and sarcosine fit into the binding site.

Although the neighboring residues (Cys-306 in GLYT1b, Ala-482 in GLYT2a) also differ between GLYT1 and GLYT2, these differences do not appear to influence substrate selectivity. This is not surprising, because in the crystal structure of LeuT<sub>AA</sub> the corresponding residue, Leu-257, faces away from the substrate binding pocket. The other difference between GLYT1b and GLYT2a in close proximity to the substrate binding site (Tyr-302 in GLYT1b, Phe-478 in GLYT2a) does appear to have an impact on substrate transport. The GLYT2a-F478Y mutant was non-functional, and the GLYT1b-Y302F mutant showed reduced expression levels, reduced glycine, and sarcosine transport currents and lower EC<sub>50</sub> values for both glycine and sarcosine. In LeuT<sub>AA</sub> the corresponding residue is Phe-253, and the main chain carbonyl of this residue forms a hydrogen bond with the amino group of leucine. It is possible that the Tyr-Phe difference between GLYT1b and GLYT2a may alter the conformation of the unwound section of TM6 (residues 256–260 of LeuT<sub>AA</sub> or 305–309 in GLYT1b) or that the difference may change the way the transporter moves to either allow substrate access to the site or substrate exit from the site as part of the transport process. It is interesting to note that a mutation of human GLYT2 W482R (equivalent to W484 in the clone used in this study) has been identified as causing startle disease (23). This mutant transporter is expressed at the cell surface and binds Na⁺ ions, but does not bind glycine. Thus, this observation and the results presented in the current study suggest that TM6 plays an important role in glycine binding and transport by glycine transporters.

This study has provided an explanation for differences in substrate selectivity between glycine transporters, and we
attempted to extend these observations to see if the same changes can explain differences in blocker sensitivities. The GLYT1b-G305S mutant is not functional, but the GLYT1b-G305A transporter does transport both glycine and sarcosine and did show slightly reduced sensitivity to NFPS compared with GLYT1b (31 ± 7% inhibition for GLYT1b-G305A compared with 43 ± 3% inhibition for wild type GLYT1b). The effect was marginal, and therefore, while the hydroxyl group of the Ser residue may play a minor role in influencing blocker selectivity, it is highly likely that other regions of the transporter will also play important roles in binding the blocker.

The GLYT2 mutants S481G and S481A transport sarcosine, and therefore we tested whether the ability of the transporters to bind sarcosine was related to their ability to bind NFPS. However, the GLYT2 mutants do not show any changes in NFPS sensitivity compared with wild type GLYT2. We also investigated whether differences in sensitivity to the GLYT2 inhibitor were related to this amino acid difference. However, all GLYT2 mutants retained sensitivity to the GLYT2 inhibitor, ALX1393. These results suggest that the molecular basis for selectivity of the two glycine transport blockers do not directly correspond with the same region that confers substrate selectivity. There has been one other study on the molecular basis for blocker selectivity of glycine transporters (22). In this study it was suggested that residues within TM1 of GLYT1 contributed to the formation of the NFPS binding site. There are a number of amino acid residues in TM1 that differ between GLYT1 and GLYT2 and the E40D (GLYT1 numbering) difference appears to be partially responsible for the difference in NFPS affinity. At present it is not clear as to whether this difference causes a direct or an indirect change in the structure of the blocker binding site. Glu-40 of GLYT1 is predicted to be located near the intracellular edge of TM1 (10), which is unlikely to be accessible to the bulky NFPS molecule when the transporter is in an inactive state or when both internal and external gates are closed (as depicted in the crystal structure (10)). However, it remains to be seen how the structure of the transporter changes during the transport process and whether the structure opens up sufficiently to allow NFPS access to this site. An alternate interpretation of the role of the Glu-40 residue in GLYT1 is that this residue is important for determining the conformation of a distant site of the transporter that binds NFPS. At present, there is no further understanding of how or where ALX1393 binds to GLYT2.

In the LeuT_Aa structure, two Na^+ binding sites are identified, and on the basis of sequence similarities between LeuT_Aa, GLYT1, and GLYT2 at least one of the Na^+ sites is likely to be conserved (10). The second Na^+ in LeuT_Aa is not conserved in the glycine transporters and yet GLYT1 co-transporters 2 Na^+ ions and GLYT2 co-transporters 3 Na^+ ions, which raises the question as to where these additional Na^+ binding sites on the glycine transporters are located. One of the interesting features of the Na^+ recognition sites in LeuT_Aa is the role of polar, but uncharged, residues in coordinating ion binding, and given that the GLYT2 mutant converts the polar Ser residue to a Gly residue, we investigated whether the mutation altered the Na^+ coupling ratio. However, although the mutation altered the substrate selectivity, it did not change the Na^+ coupling ratio. Thus, we conclude that this residue difference between GLYT1 and GLYT2 does not play a role in determining differences in the Na^+ coupling ratio.

This study has tested the hypothesis of Yamashita et al. (10) that the structure of the bacterial leucine transporter, LeuT_Aa, represents a good working model for understanding the structures of the Na^+ /Cl^−-dependent glycine transporters. We have presented evidence demonstrating that the predictions from the structure of LeuT_Aa concerning substrate recognition hold true for the glycine transporters.

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