Neutralizing Aspartate 83 Modifies Substrate Translocation of Excitatory Amino Acid Transporter 3 (EAAT3) Glutamate Transporters*\(^{5}\)

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Excitatory amino acid transporters (EAATs) terminate glutamatergic synaptic transmission by removing glutamate from the synaptic cleft into neuronal and glial cells. EAATs are not only secondary active glutamate transporters but also function as anion channels. Gating of EAAT anion channels is tightly coupled to transitions within the glutamate uptake cycle, resulting in \(\text{Na}^{+}\) - and glutamate-dependent anion currents. A point mutation neutralizing a conserved aspartic acid within the intracellular loop close to the end of transmembrane domain 2 was recently shown to modify the substrate dependence of EAAT anion currents. To distinguish whether this mutation affects transitions within the uptake cycle or directly modifies the opening/closing of the anion channel, we used voltage clamp fluorometry. Using three different sites for fluorophore attachment, V120C, M205C, and A430C, we observed time-\(^{-}\), voltage-\(^{-}\), and substrate-dependent alterations of EAAT3 fluorescence intensities. The voltage and substrate dependence of fluorescence intensities can be described by a 15-state model of the transport cycle in which several states are connected to branching anion channel states. D83A-mediated changes of fluorescence intensities, anion currents, and secondary active transport can be explained by exclusive modifications of substrate translocation rates. In contrast, sole modification of anion channel opening and closing is insufficient to account for all experimental data. We conclude that D83A has direct effects on the glutamate transport cycle and that these effects result in changed anion channel function.

The abbreviations used are: EAAT, excitatory amino acid transporter; TM, transmembrane domain; HP, hairpin.

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functions, and thus, we decided to further study this mutation using voltage clamp fluorometry. Voltage clamp fluorometry combines electrophysiological and fluorescence measurements and has been used to monitor conformational changes in diverse transport proteins (21–23). We inserted the homologous mutation D83A into EAAT3 and applied voltage clamp fluorometry to separate the effects on coupled transport and on anion channel function. EAAT3 has already been studied using voltage clamp fluorometry, permitting detection of conformational rearrangements during coupled transport (23–26). Moreover, EAAT3 effectively transports glutamate (12, 27), thus permitting additional direct insights into the effects of neutralizing Asp-83 on coupled transport.

We found that D83A changes the time, substrate, and voltage dependence of EAAT3 fluorescence signals. Moreover, similar to earlier data on D112A EAAT1 and D117A EAAT4, D83A enlarged EAAT3 anion currents in the absence of glutamate but reduced glutamate-sensitive current amplitudes. It reduced coupled transport by about 50%. The data on fluorescence amplitudes and transport function can be described by a kinetic scheme in which only transitions within the uptake cycle are modified by the mutation, whereas the likelihood of anion channel opening was not changed in mutant transporters.

EXPERIMENTAL PROCEDURES

Expression of WT and Mutant hEAAT3 Transporters in Xenopus laevis Oocytes—Point mutations were introduced into pTLN2-hEAAT3 (28, 29) (kindly provided by Dr. Matthias Hediger (University of Bern, Switzerland)) using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Capped cRNA was synthesized from MluI-/NheI-linearized pTLN2-hEAAT3 through use of mMESSAGE mMACHINE kits (Ambion, Austin, TX). Collagenase-treated, defolliculated stage IV-V oocytes were microinjected with 10 ng of RNA (Nanoliter 2000, World Precision Instruments, Sarasota, FL) and incubated at 18 °C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Hepes, pH 7.6) and has been used to monitor conformational changes in more than one protein region. Moreover, EAAT3 effectively transports glutamate (12, 27), whereas V120C has not been reported to date. Because accessible endogenous cysteine, we additionally mutated Cys-158 to serine in all our constructs to prevent possible modifications by fluorophore application.

For each reporter mutation, the time course of fluorescence was calculated as the sum of the products of relative fluorescence and probability of occupation for every state. This model was then adapted to D83A EAAT3 either by modifying translocation rates (reactions 8, 15, and 17) or anion channel open probabilities. In all cases, detailed balance was preserved, and fitting parameters were simultaneously optimized against cysteine-substituted EAAT3 (23). Anion channel open probabilities were estimated by optimizing the model against cysteine-substituted WT steady-state fluorescence data using the genetic algorithm as implemented in MATLAB (The MathWorks). The overall fluorescence was calculated as the sum of the products of relative fluorescence and probability of occupation for every state. For kinetic modeling, we assumed the following intracellular concentrations: [Na+] = 10 mM, [K+] = 70 mM, [glutamate] = 12 mM, pH = 7.3 (1). Extracellular concentrations were set to experimental conditions.

RESULTS

Time, Substrate, and Voltage Dependence of EAAT-associated Currents and Fluorescence Signals—To permit site-directed fluorescence labeling of EAAT3, we introduced single cysteines into human EAAT3 and expressed WT and cysteine-substituted transporters in Xenopus oocytes (Fig. 1). We chose three reporter mutations localized in different parts of the transporter, V120C (3–4 loop), M205C (TM4c), and A430C (7–8 loop) (Fig. 1A), to observe fluorescence changes due to conformational changes in more than one protein region. M205C and A430C have already been used in earlier studies (23, 25), whereas V120C has not been reported to date. Because the fluorophore attachment site, Cys-120, is localized nearby an accessible endogenous cysteine, we additionally mutated Cys-158 to serine in all our constructs to prevent possible modifications by fluorophore application.

For each reporter mutation, the time course of fluorescence labeling was tested to define incubation times sufficient for

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maximum labeling (supplemental Fig. 1). V120C, M205C, and A430C EAAT3-injected oocytes exhibited higher levels of fluorescence intensities than un.injected oocytes or oocytes expressing C158S EAAT3 (data not shown). Moreover, oocytes expressing V120C, M205C, or A430C EAAT3 displayed fluorescence changes upon voltage or substrate alterations (supplementary Fig. 2) that were absent in un injected oocytes or those expressing C158S EAAT3, indicating that cysteine-substituted EAAT3 is specifically labeled with the fluorescent dye.

To test whether cysteine insertions change glutamate transport or anion conduction, we studied WT and cysteine-substituted EAAT3 currents using two-electrode voltage clamp (Fig. 1B). Application of glutamate resulted in a pronounced increase of current amplitudes at negative as well as at positive voltages. In the absence as well as in the presence of glutamate, current amplitudes in oocytes expressing V120C, M205C, or A430C EAAT3 were not statistically different from those of WT EAAT3, illustrating comparable expression levels. EAAT3 currents consist of two components, glutamate uptake currents due to electrogenic transport and EAAT3-associated anion currents. Glutamate uptake current can be separated from anion currents after substituting permeant anions with impermeant gluconate and measured as the difference of currents in the presence and in the absence of glutamate (2, 13, 33). Glutamate uptake is strongly voltage-dependent and decreases to values around zero at positive potentials (2). Thus, currents at positive voltages predominantly represent anion currents. The use of NO₃⁻ as the main external anion results in EAAT3 currents that are significantly larger than endogenous currents of un injected oocytes and permit accurate measurements of EAAT3 anion currents (Fig. 1B) (33). For all tested constructs, glutamate uptake currents and anion currents at saturating glutamate concentrations were not statistically different from WT values, illustrating that none of the cysteine substitutions modify functional properties of EAAT3 (23, 25) (Fig. 1, B and C).

Fig. 2 shows representative fluorescence recordings from V120C, M205C, or A430C EAAT3 in the absence (top) or in the presence (bottom) of 1 mM L-glutamate. Oocytes were held at 0 mV, and voltage steps between −150 mV and +150 mV were applied in 50-mV intervals. In the absence of glutamate, hyperpolarizing voltage steps caused a slow reduction of V120C EAAT3 fluorescence that could be fit with a monoexponential function (τ = 46.7 ± 4.3 ms, n = 3) (Fig. 2A). M205C and A430C EAAT3 exhibited fluorescence amplitudes that, in the absence of glutamate, increased upon membrane depolarization and decreased upon hyperpolarization (Fig. 2, B and C). The time course of these voltage-dependent changes was significantly faster than for V120C (τ = 0.4 ± 0.1 ms, n = 3; τ = 0.6 ± 0.1 ms, n = 3) (Fig. 2A). For M205C and A430C EAAT3, the initial rise upon membrane depolarization was followed by a much slower decay (Fig. 2).

Application of glutamate increased fluorescence intensities of V120C EAAT3 and decreased these values for M205C and A430C EAAT3. Glutamate modified the voltage dependences of fluorescence signals for all cysteine-substituted EAAT3 constructs (Fig. 2). Application of L-glutamate accelerated the time course of fluorescence change (τ = 16 ± 0.5 ms, n = 3) and modified its voltage dependence for V120C EAAT3 (Fig. 2A). For M205C and A430C EAAT3 (Fig. 2, B and C), fluorescence showed the lowest intensity around 0 mV and increased upon positive as well as negative membrane potentials. In the presence of glutamate, fluorescence increased on a slow time
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EAAT4 anion channel gating depends on anion concentrations on both membrane sites (19, 33). To test whether EAAT3 fluorescence intensities undergo anion-dependent alterations, we studied all cysteine-substituted EAAT3 constructs after substitution of the external anion by gluconate. Chloride removal did not change the intensity or voltage dependence of fluorescence signals (supplemental Fig. 3, A–C). We conclude that fluorescence changes of fluorophores added to V120C, M205C, and A430C report on conformational changes within the EAAT3 uptake cycle that require external Na\(^+\) but do not depend on the anion composition.

Voltage Clamp Fluorometry Reveals Additional Slow Conformational Changes—In addition to the initial rise upon depolarization, fluorescence intensities of M205C EAAT3 exhibited voltage-dependent changes with a very slow time course. Fig. 3 shows such current and fluorescence recordings of M205C EAAT3 on an extended time scale. In the absence of glutamate, a depolarizing voltage step to +50 mV caused a very slow fluorescence decay (\(\tau_{(+50 \text{ mV})} = 6.7 \pm 1.2 \text{ s}, n = 6\)). This process is not due to bleaching because we observed slow fluorescence recovery upon return to the holding potential of 0 mV (Fig. 3A). Application of glutamate shifted the voltage dependence of these slow processes so that relaxation of these signals was observed upon voltage steps to –100 mV (\(\tau_{(-100 \text{ mV})} = 4.0 \pm 1.2 \text{ s}, n = 11\)) (Fig. 3A).

These fluorescence changes are too slow to represent transitions within the uptake cycle. However, we did not observe slow fluorescence changes in the absence of transport substrates or in the presence of dl-threo-benzyloxyaspartate (35, 36), inhibited fluorescence changes in the absence and in the presence of glutamate by about 90%, supporting the notion that fluorescence changes are due to conformational rearrangements in the glutamate transporter (supplemental Fig. 2).

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EAAT-mediated glutamate transport is based on a series of substrate association steps and related conformational changes. Three Na\(^+\) ions, one H\(^+\), and one glutamate associate with the outward facing transporter with an open extracellular gate (hairpin 2 (HP2)). After closure of HP2 and movement of the translocation domain to the inside (34), Na\(^+\), H\(^+\), and glutamate are released by opening of the intracellular gate (hairpin 1 (HP1)), and the uptake cycle is completed by retranslocation after association of K\(^+\) or Na\(^+\). Modification of the uptake cycle by removal of certain substrates or application of EAAT-specific blockers allows testing of the specificity of the observed signals. For all constructs, substitution of Na\(^+\) by choline\(^+\) abolished the voltage dependence of fluorescence signals and changed the fluorescence intensity (Fig. 2). A specific blocker of EAAT transporters, DL-threo-benzyloxyaspartate (35, 36), inhibited fluorescence changes in the absence and in the presence of glutamate by about 90%, supporting the notion that fluorescence changes are due to conformational rearrangements in the glutamate transporter (supplemental Fig. 2).

**FIGURE 2. Voltage- and substrate-dependent conformational changes of EAAT3.** Representative fluorescence traces (left column) recorded from X. laevis oocytes expressing V120C (A), M205C (B), and A430C EAAT3 (C) and averaged voltage dependence of late (arrow) relative fluorescence changes (right column) in the presence of different substrates are shown. Fluorescence signals were measured in NaCl Ringer’s solution during 200-ms voltage steps between +150 and −150 mV. Fluorescence amplitudes were normalized to the fluorescence generated at +150 mV in NaCl solution. Measurements in NaCl at voltages above 0 mV are shown in red, 0 mV is in dark red, and negative voltages in the absence of 1 mM glutamate are shown in light gray and in the presence of 1 mM glutamate are shown in black. Fluorescence data from at least six oocytes are reported as means ± S.E. F, fluorescence; glu, glutamate.
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D83A Modifies EAAT3 Glutamate Transport, Anion Currents, and Fluorescence Amplitudes—We next inserted the D83A mutation into each cysteine-substituted EAAT3 construct and studied the functional effects of this mutation using voltage clamp analysis. Asp-83 is localized between TM2 and TM3 and is highly conserved among mammalian EAATs (Fig. 4A). Because homologous mutations in EAAT1 (20) and EAAT4 (19) alter glutamate transport and anion conduction, D83A might allow insights into how conformational changes underlying coupled transport open and close the anion channel.

At positive membrane potentials in the absence of glutamate, D83A EAAT3 currents were statistically larger than WT or cysteine-substituted EAAT3 currents (Fig. 4, B and C) (WT (3.7 ± 0.2 μA) compared with V120C,D83A (7.7 ± 0.3 μA), p < 0.001; compared with M205C,D83A (6.1 ± 0.5 μA), p = 0.006; and compared with A430C,D83A (9.5 ± 0.5 μA), p < 0.001; n > 10). The glutamate-induced current increase was less pronounced in D83A EAAT3 than in WT EAAT3 (Fig. 4C, top). This is due to virtually glutamate-independent anion current amplitudes (Fig. 4C, middle). Moreover, D83A reduced uptake current amplitudes to values between 30 and 50% for all reporter mutations (Fig. 4C, bottom).

Voltage clamp fluorometry revealed D83A-induced changes in the voltage and substrate sensitivity of the fluorescence signals for each fluorophore attachment site (Fig. 5). V120C,D83A and A430C,D83A EAAT3 fluorescence signals increased during depolarization and decrease during hyperpolarization (Fig. 5, A and C). However, D83A modified the time course of hyperpolarization-induced fluorescence decrease (τ(V120C,D83A, −150 mV) = 18.2 ± 2.6 ms, n = 2; τ(A430C,D83A, −150/+150 mV) = 12.7 ± 2.6/11.5 ± 2.8 ms, n = 4). The voltage dependence of M205C EAAT3 fluorescence was dramatically altered by D83A. Without external glutamate, D83A EAAT3 fluorescence amplitudes displayed minimum levels around +50 mV and increased with depolarization and hyperpolarization to nearly the same intensity as in sodium-free conditions (Fig. 5B). Furthermore, the maximum change of fluorescence intensity by applying different membrane potentials was less pronounced than for M205C EAAT3 (5% instead of 10%).

Glutamate decreased the fluorescence intensity of V120C,D83A and M205C,D83A EAAT3 and modified their voltage dependences (Fig. 5, A and B). Whereas fluorescence signals of V120C,D83A and M205C,D83A EAAT3 require external Na+, A430C,D83A EAAT3 voltage-dependent fluorescence changes seemed to be less substrate-dependent (Fig. 5C). Removal of external permeant anions did not affect the intensity or the voltage dependence of fluorescence signals of D83A EAAT3 (supplemental Fig. 3, D–F), similar to our results on EAAT3 with an aspartate at position 83.

Kinetic Modeling Reveals Changes in Glutamate Uptake Cycle in D83A EAAT3—Glutamate transport by EAATs can be described with a kinetic scheme that encompasses subsequent substrate association/dissociation and translocation steps between inward and outward facing conformations (14, 31, 37) (Fig. 6). Anion channel gating is tightly coupled to these transitions and can be represented by adding branching open anion channel states (19, 38, 39). Because the transport cycle can only proceed from closed channel states (19, 38), opening of EAAT anion channels prevents progression of the uptake cycle. Changes in the distribution between open and closed anion channel states will therefore affect the uptake cycle.

D83A could alter EAAT3 function by directly modifying substrate association/dissociation or transporter translocation rates. Alternatively, D83A might modify apparent transitions within the uptake cycle by changing anion channel opening and closing transitions. To distinguish between the two possibilities, sole modification of transitions within the uptake cycle or modification by changed anion channel open probabilities, we modeled voltage-dependent fluorescence data from WT and D83A EAAT3 with a kinetic model that is based on a scheme developed to describe EAAT3 fluorescence data (23).

We modified this model by increasing the number of different fluorescence levels for each fluorescently labeled transporter from three to four. The four fluorescence levels of our kinetic scheme correspond to four structurally distinct conformations (Fig. 6). The empty transporter resides in an outward facing state with HP2 open and the substrate binding sites exposed to the extracellular space (F_{0,open}: ToK and To). Glutamate uptake is initiated by association of three Na+ ions, one H+, and one glutamate. Association of these substrates causes closure of HP2 (F_{0, occluded}: ToNa1, ToNa2, ToNa2G, ToNa2H, ToNa2GH, and ToNa3GH), and a large piston-like movement of the “translocation domain” results in the inward facing conformation (F_{1, occluded}: TiNa3GH, TiNa2GH, TiNa2G, TiNa2,
and TiNa1) (26). Opening of HP1 is followed by substrate release and exposure of the binding sites to the intracellular solution (Figopen: Ti and TiK) (23). Retranslocation after association of internal K\(^{+}\)/H\(^{+}\) completes the uptake cycle. The gating of EAAT anion channels can be represented by adding branching open channel states from the uptake cycle (Fig. 6, A and B) (19, 26).

**FIGURE 4.** D83A changes substrate dependence of EAAT3 current amplitudes. A, localization of the mutation D83A in the transmembrane topology model. The trimerization domain is shown in gray, and the transport domain is shown in black. The amino acid sequence alignment of GltPh and EAAT1–5 shows the TM2-TM3 region. Asp-83 of EAAT3 is highlighted in black. B, representative current traces recorded from *X. laevis* oocytes expressing V120C,D83A, M205C,D83A, or A430C,D83A EAAT3 and averaged voltage dependence of late (arrow) relative fluorescence changes (right column) in the presence of different substrates are shown. Fluorescence signals were measured in NaCl Ringer’s solution during 200-ms voltage steps between +150 and −150 mV. Fluorescence amplitudes were normalized to the fluorescence generated at +150 mV in NaCl solution. Measurements in NaCl at voltages above 0 mV are shown in red, 0 mV is shown in dark red, and negative voltages in the absence of 1 mM glutamate are shown in light gray and in the presence of 1 mM glutamate are shown in black. Fluorescence data from at least six oocytes are reported as means ± S.E. F, fluorescence; glu, glutamate.

**FIGURE 5.** Voltage- and substrate-dependent conformational changes of D83A EAAT3. Representative fluorescence traces (left column) recorded from *X. laevis* oocytes expressing V120C,D83A (A), M205C,D83A (B), and A430C,D83A EAAT3 (C) and averaged voltage dependence of late (arrow) relative fluorescence changes (right column) in the presence of different substrates are shown. Fluorescence signals were measured in NaCl Ringer’s solution during 200-ms voltage steps between +150 and −150 mV. Fluorescence amplitudes were normalized to the fluorescence generated at +150 mV in NaCl solution. Measurements in NaCl at voltages above 0 mV are shown in red, 0 mV is shown in dark red, and negative voltages in the absence of 1 mM glutamate are shown in light gray and in the presence of 1 mM glutamate are shown in black. Fluorescence data from at least six oocytes are reported as means ± S.E. F, fluorescence; glu, glutamate.

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To obtain a kinetic scheme describing fluorescence values, anion currents, and uptake currents for WT EAAT3, we used the same rate constants of the uptake cycle as determined in earlier work on cysteine-substituted EAAT3 (23). We determined anion channel open probabilities by fitting the model to the experimentally observed fluorescence of V120C, M205C, and A430C EAAT3. The parameters were constrained to correctly describe experimentally determined relative glutamate-induced current amplitudes at +100 mV and to preserve detailed balance (Fig. 7G). The so-obtained fitting parameters (Tables 1, 2, and 3) demonstrated a good agreement of the simulated and observed fluorescence-voltage relationship under different substrate conditions (Fig. 7, A–C). However, they predicted a transport rate of about four transported glutamates per second at −100 mV (Fig. 7F), which is lower than in previously published data relating to EAAT1, -2, or -3 (27, 40). We did not succeed in modifying fit parameters to increase transport rates. All attempts impaired the compatibility with the experimentally observed voltage dependence of fluorescence. Because unitary transport currents are extremely difficult to measure directly, we accepted this deviation between published and predicted results and only used the relative decrease of glutamate uptake currents by D83A in the optimization procedure.

We then modified the thus established kinetic model to account for the fluorescence signals and transport properties of D83A EAAT3. We initially tested whether modifying reactions in certain parts of the uptake cycle can qualitatively account for the observed D83A-mediated changes in fluorescence. There were two processes that resulted in such alterations, changes in anion channel open probability and changes in translocation rates.

We first optimized all anion channel opening/closing reactions simultaneously against fluorescence intensities for all three reporter mutations combined with D83A. Glutamate-induced increases of anion current amplitudes at +100 mV, relative glutamate uptake currents at −100 mV, and detailed balance were again used as constraints. During optimization of opening and closing rates of anion channels, global fitting yielded a set of parameters that well reproduced the fluorescence-voltage relationship of D83A EAAT3 under different ionic conditions. However, these changes always predicted reduction of glutamate transport rates to negligible values. Therefore, we evaluated whether alterations of translocation rates within the uptake cycle can account for D83A fluorescence and function. In these fitting procedures, only the three translocation reactions were adjusted. The fitting procedure revealed altered translocation rates of the K+-bound (reaction 15), the Na+-bound (reaction 17), and the Na+- and glutamate-bound transporter (reaction 8). Whereas inward translocation from ToNa3GH to TiNa3GH was promoted, D83A stimulated the outward translocation for the two other processes (Tables 1–3). These alterations resulted in predicted fluorescence data that resemble experimental data on D83A EAAT3 (Fig. 7, D–F, and Tables 1–3).

The kinetic model permits the prediction of the steady-state probability that the transporter resides in a given state of the transport cycle. We next calculated the residence probability of each state of the uptake cycle under steady-state conditions for the cysteine-substituted WT (Fig. 7f) or D83A EAAT3 (Fig. 7f). In the presence as well as in the absence of glutamate, D83A EAAT3 was predicted to reside with higher likelihood in the inward facing conformation than cysteine-substituted EAAT3.

**DISCUSSION**

Here, we studied the functional consequences of neutralizing a conserved aspartate at position 83 in EAAT3. Homologous mutations have already been studied in EAAT1 and EAAT4 (19, 20) and were reported to result in dramatic alterations of EAAT anion channel opening and closing for both isoforms. Investigating the effects of this point mutation on conformational changes promised insights into the interaction of transporter and anion channel function of EAAT glutamate transporters.

In our experiments, transporter proteins were labeled with fluorophores, and fluorescence intensities were measured upon changes in membrane voltages or in substrate concentrations.
We used three different cysteine substitutions, V120C, M205C, and A430C, for these experiments. Labeling of cysteine-substituted EAAT3 was specific because the same procedures on oocytes expressing EAAT3 that lack cysteines for fluorophore attachment resulted in background fluorescence levels (supplemental Figs. 1 and 2). For each reporter mutant, voltage steps caused changes in fluorescence amplitudes that were modified by concentrations of transporter substrates, such as Na\(^{+}\)/H\(^{+}\) and glutamate (Fig. 2). Moreover, application of DL-threo-benzyloxyaspartate abolished our observed fluorescence changes (supplemental Fig. 2). Because DL-threo-benzyloxyaspartate is known to lock EAATs into an outward facing conformation with HP2 open (35) and thus to prevent substrate binding and subsequent voltage-dependent conformational changes, this result further supports the notion that fluorescence signals arise from conformational changes of the EAAT3 transporter.

Because EAATs are dual function proteins, the observed fluorescence changes might report on transitions within the uptake cycle or on anion channel opening and closing. We found that the voltage and substrate dependence of EAAT3 fluorescence signals can be well described by a kinetic model that is based on the glutamate transport cycle and in which opening and closing transitions of anion channels are voltage-independent (Fig. 6) (5, 23, 34). Moreover, fluorescence is modified by transport substrates that affect the transport cycle but do not directly affect the anion channel opening and closing (42). Taken together, these results indicate that the observed

FIGURE 7. Simulated voltage- and substrate-dependent conformational changes, open probabilities and transport currents of cysteine-substituted and D83A EAAT3. A–F, averaged voltage dependence of measured late fluorescence changes (fluorescence-voltage relationship) (circles) and simulated fluorescence changes (solid lines) in the absence (gray) or in the presence of 1 mM glutamate (black). Fluorescence amplitudes were normalized to the fluorescence generated at +150 mV in NaCl solution. G and H, simulated open probabilities (G) at +100 mV of cysteine-substituted WT and D83A EAAT3 without (gray) and in the presence of glutamate (black) and simulated transport currents (H) at −100 mV from the data given in Tables 1 and 3. e, elementary change; e = 1.602 176 462(63) × 10\(^{-19}\). I and J, simulated residence probability of cysteine-substituted WT (I) and D83A EAAT3 (J) calculated from the data given in Table 1 without (gray) and in the presence of glutamate (black) for three different voltages, −150 (solid line), 0 (dashed line), and +150 mV (dotted line). glu, glutamate; F, fluorescence.
TABLE 1
Parameters of EAAT3 model
| Reaction | Forward | Backward | zβ |
|----------|---------|----------|----|
| 1        | 1.00 × 10^(-3) s^(-1) | 2.50 × 10^(-3) s^(-1) | 0.20 |
| 2        | 1.00 × 10^(-3) s^(-1) | 2.50 × 10^(-3) s^(-1) | 0.20 |
| 3        | 6.00 × 10^(-10) M^(-1) s^(-1) | 3.00 × 10^(-10) M^(-1) s^(-1) | -0.40 |
| 4        | 6.00 × 10^(-11) M^(-1) s^(-1) | 7.00 × 10^(-11) M^(-1) s^(-1) | 0.40 |
| 5        | 6.00 × 10^(-12) M^(-1) s^(-1) | 7.00 × 10^(-12) M^(-1) s^(-1) | 0.40 |
| 6        | 6.00 × 10^(-13) M^(-1) s^(-1) | 3.00 × 10^(-13) M^(-1) s^(-1) | 0.40 |
| 7        | 1.00 × 10^(-3) s^(-1) | 1.00 × 10^(-3) s^(-1) | 0.55 |
| 8        | 5.50 × 10^(-3) s^(-1) | 5.00 × 10^(-3) s^(-1) | 1.00 × 10^3 s^(-1) |
| 9        | 8.00 × 10^(-2) s^(-1) | 4.00 × 10^(-2) M^(-1) s^(-1) | 6.59 s^(-1) |
| 10       | 6.00 × 10^(-1) s^(-1) | 9.00 × 10^(-10) M^(-1) s^(-1) | 0.45 |
| 11       | 3.00 × 10^(-3) s^(-1) | 4.00 × 10^(-3) s^(-1) | 0.45 |
| 12       | 5.00 × 10^(-3) s^(-1) | 2.00 × 10^(-3) M^(-1) s^(-1) | 0.20 |
| 13       | 4.00 × 10^(-3) s^(-1) | 1.00 × 10^(-3) M^(-1) s^(-1) | 0.20 |
| 14       | 1.00 × 10^(-3) s^(-1) | 1.00 × 10^(-3) s^(-1) | 1.00 × 10^(-1) s^(-1) |
| 15       | 50 s^(-1) | 5.00 s^(-1) | 1.00 × 10^(-1) s^(-1) |
| 16       | 8.00 × 10^(-2) s^(-1) | 1.00 × 10^(-2) M^(-1) s^(-1) | 19.19 s^(-1) |
| 17       | 0.80 s^(-1) | 1.00 × 10^(-2) s^(-1) | 19.19 s^(-1) |

TABLE 2
Fluorescence intensities used for simulations of cysteine-substituted wild type and mutant EAAT3 fluorescence-voltage relationships

| Fluorescence state | V120C | M205C | A430C | V120C,D83A | M205C,D83A | A430C,D83A |
|--------------------|-------|-------|-------|------------|------------|------------|
| F0_open            | 1.00  | 1.00  | 1.00  | 1.00       | 1.00       | 1.00       |
| F0_closed          | 0.98  | 0.89  | 0.96  | 0.97       | 0.92       | 0.96       |
| F1_open            | 1.32  | 0.29  | 0.79  | 0.98       | 0.88       | 0.97       |
| F1_closed          | 0.52  | 2.44  | 1.25  | 0.97       | 0.88       | 0.93       |

TABLE 3
State-specific probabilities for channel opening used in kinetic modeling of cysteine-substituted wild type and mutant EAAT3

| Anion channel state branching from | Open probability |
|-----------------------------------|------------------|
| ToNa1                             | 0.722            |
| ToNa2                             | 9.999 × 10^-5    |
| ToNa2G                            | 9.999 × 10^-5    |
| ToNa2H                            | 9.999 × 10^-5    |
| ToNa2GH                           | 1.321 × 10^-4    |
| ToNa3G                            | 0.988            |
| TiNa3G                            | 0.826            |
| TiNa2GH                           | 0.713            |
| TiNa2G                            | 1.275 × 10^-4    |
| TiNa2                             | 1.009 × 10^-4    |
| TiNa1                             | 0.765            |
| TiK                               | 1.357 × 10^-4    |

fluorescence changes are not directly linked to opening and closing of the anion channel.

Time courses of fluorescence changes are therefore expected to reflect rate constants of transitions between individual states of the uptake cycle. However, we observed different kinetics but very similar voltage dependences of fluorescence changes of M205C and A430C (Fig. 2, B and C). The time dependences of fluorescence amplitudes obviously depend not only on global conformational changes of the EAAT proteins but also on additional region-specific changes of the environment. We therefore did not use the time dependence of fluorescence amplitudes to deduce information about rate constants within the uptake cycle.

For M205C EAAT3, we observed very slow changes in the fluorescence amplitude upon depolarizing voltage steps in the absence of external glutamate and upon membrane hyperpolarization after application of glutamate (Fig. 3). The time dependence of this process can be well described with a single time constant of around 7 s without glutamate and around 4 s in the presence of the substrate. Turnover rates of individual EAAT3 proteins have been determined to be well above 10/s (27), and such slow time constants thus indicate a conformational change outside the transport cycle. Because ultraslow fluorescence changes are voltage-dependent (Fig. 3A) and because glutamate modifies this voltage dependence, one might speculate about the existence of a branching connection from one of the states of the uptake cycle. After entering such a branching state, the transport cycle is interrupted and can only resume after having left this particular long lasting state. We tested the existence of such a branching connection by measuring time-dependent changes in glutamate transport currents. The existence of long lasting branching states requires that glutamate transport rates are changed with the same time constant as the fluorescence signal. However, we did not observe any time- or voltage-dependent changes in the glutamate-dependent current, indicating that there are no branching connections from one of the uptake cycle states of the transporter (Fig. 3). Slow fluorescence changes are thus due to conformational changes that occur in certain states of EAAT3 but do not interfere with the uptake cycle. At present, we can only speculate about the molecular basis and functional consequences of this finding. A possible explanation of these slow processes might be large collective motions as recently reported by Jiang et al. (43). An alternative assumption is that these very slow fluorescence changes correspond to a slow gating process within the EAAT anion channel. This gating process has been initially described
in EAAT4 where slow changes in the anion to cation selectivity occur upon membrane depolarization. We also observed similar processes for EAAT3, and the time and voltage dependences of these gating processes resemble the observed slow changes of the fluorescence signals (17).

Due to the slow changes of fluorescence amplitudes, it was impossible to obtain and interpret steady-state fluorescence values. We therefore determined fluorescence values at the end of a 200-ms pulse. After this time period, all processes within the uptake cycle will have reached steady-state conditions. Moreover, the additional slow processes that occur outside the uptake cycle and thus should not contribute to the measured fluorescence values will not have caused major alterations of the fluorescence levels.

We recently performed a detailed analysis of the effects of D117A on EAAT4 anion currents (19). Noise analysis demonstrated reduced unitary current amplitudes of D117A EAAT4 anion channels, and reversal potential measurements under bionic conditions indicated altered anion selectivity. These functional alterations supported direct modification of the EAAT4 anion conduction pathway by D117A. We reasoned that Asp-117 is in close proximity to the anion conduction pathway and that D117A alters anion channel open probabilities by directly modifying opening and closing transitions. We optimized rate constants in the kinetic scheme that is closely similar to the one shown in Fig. 6 against experimentally determined absolute open probabilities for WT and D117A EAAT4 at different voltages. This procedure revealed that the effect of D117A on EAAT4 gating can be well reproduced by exclusive modification of anion channel open probabilities.

We now studied the effects of the homologous mutation on EAAT3 using voltage clamp fluorometry. The use of EAAT3 permits direct measurements of glutamate uptake currents, and this parameter turned out to be crucial for our interpretation of the effects of D83A. Cysteine-substituted EAAT3 fluorescence, glutamate transport, and anion currents can be well represented by a kinetic scheme that is based on a model developed in a recent voltage clamp fluorometry study (23). We optimized this scheme by inserting anion channel states branching from certain states of the glutamate transport cycle. For WT EAAT3, rate constants within the uptake cycle were not modified. The glutamate transport cycle consists of many different steps, and some of them are outside the temporal resolution of our system. Moreover, the kinetic scheme encompasses too many rate constants to be accurately determined in separation. The resulting scheme is in good agreement with all our experimental data on WT. However, there is one published feature of EAAT3 for which the kinetic model fails to account. It is not able to correctly predict the high transport rates of EAAT3 for reverse transport at high external K\(^+\) (44). Because our experiments were performed in the absence of external K\(^+\), we accepted this limitation of the kinetic scheme.

The effects of D83A on fluorescence and anion current could be explained by modifying anion channel gating. However, these alterations predicted a reduction of the glutamate uptake rates to very low values and are thus in disagreement with experimental data (Fig. 4). Only by modification of translocation rates in the uptake cycle were we able to accurately describe all existing data, glutamate transport, anion currents, and fluorescence data. Our data thus indicate that D83A modifies translocation rates. These changes in translocation are supported by available crystal structures that place the homologous residue of Asp-83/Asp-117 to the TM2-TM3 loop between the trimerization and transport domains. By altering the hinge function of this region (19, 34, 45), D83A/D117A might affect the isomerization between inward and outward facing conformations.

The different outcome of the two studies on EAAT3 and EAAT4 is most likely not due to isoform-specific differences in anion channel function but rather caused by the separate experimental approaches. Anion channel properties are very well conserved between EAAT3 and EAAT4 with closely similar unitary conductance, selectivity, and open probabilities (33). EAAT4 differs from EAAT3 in very low glutamate transport rates (27). Changes in translocation processes might have less influence on anion channel gating in EAAT4 than in EAAT3. D83A is thus expected to exert effects on EAAT3 anion channels similar to those of D117A on EAAT4 anion channels. We conclude that D83A/D117A modifies substrate translocation and anion channel opening as well as anion conduction by EAAT3/EAAT4.

At present, the molecular basis of anion conduction in EAAT glutamate transporters is insufficiently understood. So far, only one point mutation, D117A, has been shown to modify unitary anion conduction rates in the outward (35) as well as in the inward facing conformation (34). Ser-74, the Glu\(_{\text{tm}}\) residue homologous to Asp-83/Asp-117, projects into surrounding lipids rather than into possible conduction pathways. Moreover, D117A reduces unitary current amplitudes of EAAT4 anion channels by removing a negative charge. These two findings argue against a direct effect of D83A/D117A on anion conduction. A possible scenario that accounts for the effects of D83A on substrate translocation in EAAT3 and of D117A on anion conduction in EAAT4 is that anion channel formation is closely related to translocation. One might speculate that the anion pore opens upon moving the translocation domain between outward to inward facing conformations. Indeed, a recently reported high resolution structure of an intermediate conformation of Glu\(_{\text{tm}}\) revealed an aqueous conduction pathway that might permit permeation of anions (32). By altering the translocation trajectory, D83A/D117A could not only affect substrate translocation and anion channel opening/closing but also the anion conduction pathway itself.

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