Molecular Dynamics Simulations Elucidate the Mechanism of Proton Transport in the Glutamate Transporter EAAT3

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ABSTRACT  The uptake of glutamate in nerve synapses is carried out by the excitatory amino acid transporters (EAATs), involving the cotransport of a proton and three Na\(^+\) ions and the countertransport of a K\(^+\) ion. In this study, we use an EAAT3 homology model to calculate the pK\(_a\) of several titratable residues around the glutamate binding site to locate the proton carrier site involved in the translocation of the substrate. After identifying E374 as the main candidate for carrying the proton, we calculate the protonation state of this residue in different conformations of EAAT3 and with different ligands bound. We find that E374 is protonated in the fully bound state, but removing the Na\(_2\) ion and the substrate reduces the pK\(_a\) of this residue and favors the release of the proton to solution. Removing the remaining Na\(^+\) ions again favors the protonation of E374 in both the outward- and inward-facing states, hence the proton is not released in the empty transporter. By calculating the pK\(_a\) of E374 with a K\(^+\) ion bound in three possible sites, we show that binding of the K\(^+\) ion is necessary for the release of the proton in the inward-facing state. This suggests a mechanism in which a K\(^+\) ion replaces one of the ligands bound to the transporter, which may explain the faster transport rates of the EAATs compared to its archaeal homologs.

INTRODUCTION

Excitatory amino acid transporters (EAATs) are essential for proper functioning of the nervous system because they clear the excess glutamate released at the nerve synapses. Glutamate can be toxic to neurons in large concentrations, so problems in these transporters have been implicated in many pathological conditions including cerebral ischemia, amyotrophic lateral sclerosis, and Alzheimer’s disease (1). EAATs transport the substrate across the membrane by coupling to three Na\(^+\) ions and a proton, and a K\(^+\) ion is countertransported at every cycle. There is a great amount of experimental data on EAATs, mostly through mutagenesis experiments (4–26), but also via structural information (27,28) and the pH dependence of transport (19,29). A significant step in our understanding of the EAATs came with the solution of the crystal structures of the archaeal homolog Glt\(_{ph}\) both in the outward- and inward-facing conformations (30–32). Glt\(_{ph}\) has aspartate as the main substrate, and the coordination of the Na\(^+\) ions and the substrate in Glt\(_{ph}\) are conserved in the EAATs (23,30–32,34,35). We have shown that the model is stable with glutamate as a ligand, and that the coordination of the ligands can be reproduced in good agreement with mutagenesis experiments (36). A major result from this work is that the substrate is not stably bound unless the E374 side chain of EAAT3 is protonated, making this residue a natural candidate for the proton carrier, which also agrees well with experiments (19). Using the inward-facing state of EAAT3 from our model, we have also investigated three possible binding sites for the K\(^+\) ion, and found a high-affinity site that overlaps with the Na1 and Na3 binding sites.

Even though there is strong theoretical and experimental evidence pointing to the residue E374 as the proton carrier, the mechanism of proton association and dissociation from this residue in the outward and inward states of EAAT3 remains unknown. Furthermore, the protonation states of other titratable residues close to the binding site has not been thoroughly investigated, and in MD simulations, it is usually assumed that they retain the same protonation states as in solution. Here, we use homology models of EAAT3 to address these issues. We perform free energy perturbation (FEP) calculations to obtain the pK\(_a\) values of different residues close to the substrate binding site in the fully bound.
outward conformation. After identifying the residue E374 as the only one being protonated under these conditions, we calculate the p$K_a$ value of this residue in different states of the transporter and with different ligands bound. We show that the protonation/deprotonation of E374 is coupled to the binding/unbinding of the substrate, as well as the closure/opening of the HP1 and HP2 gates that give the substrate access to the solution. We also show that the binding of a K$^+$ ion in the inward state of EAAT3 is necessary for the deprotonation of E374, so that only the K$^+$ ion is transported back to the extracellular media in the second half of the transport cycle.

METHODS

Model system and simulation details

For the outward- and inward-facing conformations of EAAT3 we used the same models created in our previous work (36), built from their respective GltPh templates (Protein Data Bank (PDB) IDs 2NWX and 3KBC, respectively) and equilibrated in the presence and absence of the various ligands in both states. The GltPh templates have the substrate binding site occluded from the solvent, with an aspartate and two Na$^+$ ions bound (Na1 and Na2). To build the models, we have used the program MODELLER (37) and the alignment from (38). The templates had Asp, Na1, Na2, and Na3 bound, with the last Na$^+$ added at the binding site as described in (6). We have created the models including the ligands to obtain EAAT3 structures in the fully bound states. EAAT3 has a sequence of around 50 residues and protein atoms are fixed and the system is equilibrated with 1 atm pressure coupling is applied only in the $x$ and $y$-directions (typical of the dimensions of the simulation are 113 x 113 x 74 Å$^3$). In the second stage, the protein is gradually relaxed by reducing the restraints in several steps during MD simulations lasting 2.4 ns. We then perform a further 15 ns of simulations with no restraints applied.

MD simulations are performed using the NAMD program (42) with the CHARMM36 force field (43). The temperature is maintained at 300 K using Langevin damping with a coefficient of 5 ps$^{-1}$, and the pressure is kept at 1 atm using the Langevin piston method with a damping coefficient of 20 ps$^{-1}$ (44). Periodic boundary conditions with the particle mesh Ewald method are employed to calculate the electrostatic interactions without truncation. The Lennard-Jones interactions are switched off between 10 and 12 Å using a smooth switching function. A time step of 2 fs is used in all MD simulations.

Free energy calculations

To calculate the p$K_a$ values of the aspartate and glutamate side chains in the vicinity of the glutamate binding site, we use the alchemical FEP and thermodynamic integration (TI) methods in MD simulations (45). These methods use an explicit solvent and have advantages over continuum electrostatic models. Continuum models are very sensitive to the dielectric constant assigned to the protein interior, and the value used is usually an empirical parameter with a value ranging from 8 to 20 to account for different effects such as water penetration, protein flexibility, and conformational rearrangements in response to the ionization state (46–48). These models are also influenced by the method used in constructing the dielectric boundary between the protein and the surrounding environment (49). All these effects can be taken into account rigorously by computing the free energy difference between the protonated and deprotonated forms using the MF/FEP method with an explicit solvent (50). This is carried out by alchemically transforming a deprotonated carboxyl group to a protonated one in the protein while simultaneously performing the opposite transformation in a reference residue located in bulk solvent, according to the thermodynamic cycle shown in Fig. 1. The reference residues, or model compounds, that we use are N-acetyl-L-isosaparagine for the aspartate calculations and the N-acetyl-L-isoglutamine for the glutamate calculations (Fig. 2). These two compounds reproduce the side chains of aspartate and glutamate and also the backbone atoms, but do not have the zwitterion charges that could affect the electrostatic component of the results. The experimental p$K_a$ values of N-acetyl-L-isosaparanagine and N-acetyl-L-isoglutamine are 4.0 and 4.3, which compare well with the p$K_a$ values of aspartate (3.7) and glutamate (4.3) (51). The CHARMM parameters are readily available for these compounds, which further facilitate their use in FEP calculations. The p$K_a$ value of a residue in a protein is given by (32):

$$pK_a = pK_{a\text{model}} - \frac{\Delta \Delta G_{pF}}{2.3k_BT}$$

where $\Delta \Delta G_{pF}$ can be calculated from the thermodynamic cycle in Fig. 1.

FIGURE 1 The thermodynamic cycle used in the FEP/TI calculations: $A$ represents the side chain of interest, $H$ is the proton, the indices $p$ and $d$ refer to the protonated and deprotonated states, and the subscripts $m$ and $p$ indicate that $A$ is in the bulk solvent (model compound) and in the protein, respectively.
FIGURE 2  The model compounds used in our free energy calculations in bulk. (A) N-acetyl-L-isoasparagine is used for aspartate. (B) N-acetyl-L-isoglutamine is used for glutamate. To see this figure in color, go online.

\[
\Delta \Delta G_{pr} = \Delta G_{pr-m}(d) - \Delta G_{pr-m}(p) = \Delta G_{pr}(d \rightarrow p) - \Delta G_{m}(d \rightarrow p). \tag{2}
\]

MD/FEP calculations are performed to determine the free energy difference on the right side of Eq. 2, corresponding to the horizontal legs in Fig. 1.

We use both the MD/FEP and MD/TI methods to calculate \(\Delta \Delta G_{pr}\) by alchemically transforming a deprotonated glutamate residue to a protonated one in the protein, whereas doing the opposite transformation in the model compound in bulk. This process is carried out as a function of the reaction coordinate \(\lambda\), in which the end points are at \(\lambda = 0\) and \(\lambda = 1\). In the FEP method, the interval between \(\lambda = 0\) and \(\lambda = 1\) is divided into \(n\) subintervals with \(\lambda_i, i = 1, \ldots, n\), and for each subinterval the free energy difference is calculated from the ensemble average

\[
\Delta G_i = -k_BT \ln \langle \exp \left[ - (H(\lambda_{i+1}) - H(\lambda_i))/k_BT \right]\rangle_{\lambda_i}, \tag{3}
\]

where \(H(\lambda) = (1-\lambda)H_0 + \lambda H_1\), with \(H_0\) and \(H_1\) representing the states with the proton in the model compound and in the protein residue, respectively. The free energy difference is obtained from the sum, \(\Delta \Delta G_{pr} = \sum \Delta G_i\). In the TI method, the ensemble average of the derivative, \(\partial H(\lambda)/\partial \lambda\), is obtained at several \(\lambda\) values, and the free energy difference is calculated from the integral:

\[
\Delta \Delta G_{pr} = \int_0^1 \left\langle \frac{\partial H(\lambda)}{\partial \lambda} \right\rangle_{\lambda} d\lambda. \tag{4}
\]

Provided the integrand can be fitted well with a polynomial, Gaussian quadrature provides an efficient and accurate method for evaluation of such integrals because it allows for longer sampling of a smaller number of windows. In the FEP simulations we use 66 exponentially spaced windows with 40 ps of equilibration plus 40 ps of production for each window. In the TI method, we use seven windows starting from the nearest FEP windows, and run 0.4 ns of equilibration plus 0.8 ns of data collection for each window. These parameters have been found to be optimal in previous studies of GltpH (53,54). We report here only the TI results because they show better convergence, but we always check for consistency between the two methods, as well as performing both the forward and backward transformations in each case to check against any hysteresis effects. It is important to assess the convergence of \(pK_a\) calculations and determine the associated uncertainty (55). Evidence for the convergence of the TI results and the consistency between the TI and FEP results are presented in Fig. S1 in the Supporting Material. The statistical errors shown in the results are obtained by performing block data analysis over 100 ps blocks.

RESULTS AND DISCUSSION

Evaluating the model

We have assessed the quality the open-state EAAT3 model obtained from GltpK using three different methods, similar to the case of the EAAT3 models obtained from the closed states of GltpH (36). QMEAN (Qualitative Model Energy ANalysis) is a composite scoring function ranging from 0 to 1, which describes the major geometrical aspects of protein structures (39). DFIRE (Distance-scaled, Finite Ideal-gas REference) is a potential based on a database of nonhomologous proteins (38). DOPE (Discrete Optimized Protein Energy) is an atomic distance-dependent statistical potential calculated from a sample of native structures (37). The first two are available on the Swiss Model Server, and the last one is built into the program MODELER. The scores obtained for the EAAT3 models based on the GltpH templates can be found in (36). In Table 1 we show the QMEAN scores and DFIRE energies for the original GltpK crystal structure, as well as our EAAT3 model in the outward apo state. As in the case of GltpK (36), the QMEAN scores and DFIRE energies are very similar, as well as the energy profiles obtained using DOPE (Fig. 3). This indicates that, even though the homology is not high between the archaeal and the human transporters, we can still build high quality models using the former as a template. Furthermore, we are looking at ligand binding and small conformational changes instead of large conformational transitions. Therefore, the models built for the EAATs in the vicinity of the outward- and inward-facing states are likely to retain the same features of the archaeal transporters in the same states.

\[\text{pK}_a\] values in the glutamate-bound outward EAAT3

The first step in identifying the proton carrier is to calculate the \(\text{pK}_a\) values of all titratable residues in the vicinity of the binding pocket. The fully bound outward closed state

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of the QMEAN scores and DFIRE energies between the GltpK crystal structure and the EAAT3 homology model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>QMEAN score</td>
</tr>
<tr>
<td>GltpK (4KY0)</td>
<td>0.515</td>
</tr>
<tr>
<td>EAAT3 (outward apo)</td>
<td>0.484</td>
</tr>
</tbody>
</table>

The QMEAN score goes from 0 to 1, 1 being the best possible model. Lower DFIRE energy values indicate better quality structures.
precedes the transition to the inward-facing transporter, and therefore should have a proton bound. We show this structure with all ligands bound in Fig. S2A. We choose this equilibrated model to look for the proton carrier in the EAATs, by performing pKₐ calculations as described in Methods. We have shown previously that the substrate is not stable unless the E374 residue—which is a glutamine in GltPh and GltTk—is protonated (36). We cannot discard the possibility that other residues in the binding pocket could also be protonated, therefore, in addition to E374, we have also calculated the pKₐ values of the residues D368, D440, D444, and D455. The side chains of D455 and D368 are involved in Na⁺ binding at sites Na1 and Na3, the side chain of D444 coordinates the substrate α-amino group, and the side chain of D440 makes a salt bridge with the R447 residue (Fig. 4). This arginine also coordinates the glutamate substrate side-chain carboxyl group. To test the suitability of our reference residues in bulk, we have also calculated the pKₐ values of an aspartate (D83) and a glutamate (E135) residue in the protein that are fully exposed to the solvent and are not close to any charged groups. These residues are expected to retain the same pKₐ values as in bulk. To avoid the unbinding of ligands during the protonation process, all the ligands are restrained during the pKₐ calculations at the binding sites found in (36). The harmonic restraints are applied on all the heavy atoms of the substrate, with a spring constant of $k = 0.5$ (kcal/mol)/Å² on each. Similarly, the ions are restrained with a spring constant of $k = 5$ (kcal/mol)/Å². These values allow fluctuations of the ligands around their average positions $(r_0)$, similar to the ones observed for the unrestrained ligands obtained from $k \sim 3k_B T/(r - r_0)^2$, but do not allow the ligand to leave the binding site (56). This procedure enables us to calculate the pKₐ values in a given state, so we can determine which residues are protonated under these conditions. The Na1 and Na2 ions share the same binding sites as the GltPh 2NWX crystal structure, and the Na3 site binding site was determined from MD simulations and mutagenesis experiments (6). The coordination of the glutamate substrate is very similar to that of aspartate in GltPh, which is stable with a protonated E374, and is in good agreement with the mutagenesis experiments (21,23,36).

In Table 2, we present the calculated pKₐ values of the glutamate and aspartate residues mentioned previously. The positions of the titratable residues relative to the bound ligands, with their calculated pKₐ values, are shown in Fig. S2B. The pKₐ values for the test residues D83 and E135 are 3.5 and 4.2, respectively, which are in good agreement with the experimentally determined pKₐ values for the side chains of aspartate and glutamate (3.7 and 4.3). Thus, our models can accurately reproduce the properties of the aspartate and glutamate side chains in bulk water. Returning to the EAAT3 residues, we find a pKₐ value of 19.1 for E374 (Table 2), indicating that this residue is undoubtedly protonated in the fully bound outward state of EAAT3. We believe that this large pKₐ value is related to both the presence of the glutamate substrate and the closure of the HP2 gate after this ligand binds. We have shown previously that glutamate...
TABLE 2 Calculated pK_a values of various aspartate and glutamate residues in EAAT3

<table>
<thead>
<tr>
<th>Residue</th>
<th>ΔΔG_fpr</th>
<th>−ΔΔG_bch</th>
<th>ΔΔG_pr</th>
<th>ΔpK_a</th>
<th>pK_a</th>
</tr>
</thead>
<tbody>
<tr>
<td>D83</td>
<td>0.4±0.8</td>
<td>1.1±0.9</td>
<td>0.7±0.8</td>
<td>−0.5±0.6</td>
<td>3.5±0.6</td>
</tr>
<tr>
<td>E135</td>
<td>0.2±0.9</td>
<td>0.0±0.8</td>
<td>0.2±0.8</td>
<td>−0.1±0.6</td>
<td>4.2±0.6</td>
</tr>
<tr>
<td>E374</td>
<td>−20.1±0.7</td>
<td>−20.3±1.1</td>
<td>−20.2±0.9</td>
<td>14.7±0.7</td>
<td>19.1±0.7</td>
</tr>
<tr>
<td>D368</td>
<td>18.8±0.9</td>
<td>17.0±0.9</td>
<td>17.9±0.9</td>
<td>−13.1±0.7</td>
<td>−9.1±0.7</td>
</tr>
<tr>
<td>D440</td>
<td>6.8±1.7</td>
<td>6.0±1.1</td>
<td>6.4±1.4</td>
<td>−4.7±1.0</td>
<td>−0.7±1.0</td>
</tr>
<tr>
<td>D444</td>
<td>−0.4±0.9</td>
<td>0.8±1.1</td>
<td>0.2±1.0</td>
<td>−0.1±0.7</td>
<td>3.9±0.7</td>
</tr>
<tr>
<td>D455</td>
<td>16.3±2.3</td>
<td>14.9±1.5</td>
<td>15.6±1.9</td>
<td>−11.4±1.4</td>
<td>−7.4±1.4</td>
</tr>
</tbody>
</table>

The forward and the backward calculations of the protonation free energies are listed in the second and third columns, respectively, and their average in the fourth column. The shift ΔpK_a is given in the fifth column, and the final value of the pK_a is given in the last column. Errors are estimated from block data analysis using 100 ps windows. All free energies are in kcal/mol.

MD Simulations of Proton Transport in EAAT3

The substrate is not stable in the binding site if E374 is deprotonated due to the repulsion between the negatively charged carboxyl groups (36). Therefore, protonation of E374 would be energetically favorable in the presence of the substrate. Furthermore, E374 is isolated from the solvent by a nonpolar region formed by a series of hydrophobic residues at the TM7 (M367 and T370) and HP2 (A404, A408, A414, T418, and V422) segments (Fig. 5 A). All of them are strictly conserved throughout the EAAT family except for V422, which is an isoleucine in EAAT2. Conservation of this hydrophobic motif in all EAATs is indicative of its functional importance in stabilizing the protonated state of E374. Experimental studies on the ASCT1 transporter (57) show that pH sensitivity can be engineered into it by mutating the residue Q386 (equivalent to E374 in EAAT3) to glutamate. It is also found that T382 (equivalent to T370 in EAAT3) helps in stabilizing the proton bound to the E386 residue in ASCT1[Q386E], which is consistent with the results presented here. Further mutations in the hydrophobic region mentioned previously may similarly demonstrate the role of the surrounding TM7 and HP2 residues in the protonation of E374.

The experimental pK_a value of the proton carrier in EAAT3 is ~8 (29). This value is determined from the pH-dependence of the uncoupled and coupled currents associated with glutamate transport, thus reflecting the free energy difference between the fully bound protonated state and the ensemble of deprotonated conformations where the substrate translocation cannot happen. We believe that the deprotonated state of E374 is likely to be solvent exposed and without a bound substrate, given that the substrate is unstable under these conditions. Because accounting for the conformational changes and the unbinding of the substrate is not feasible in our FEP calculations, we have calculated the pK_a for the fully bound closed state, where the E374 side chain is isolated from the solvent and glutamate is bound. Hence, the calculated pK_a value of 19.1 cannot be directly compared to the experimental value, but it is sufficient to ascertain that E374 is protonated in the fully bound transporter. After 60 ns of MD simulations of EAAT3 in the absence of the substrate and the Na2 ion (36), we have observed opening of the HP2 gate and hydration of E374 (Fig. 5 B). To quantify the change in hydration of E374, we have calculated the radial distribution function of the water molecules around the carboxyl oxygens of E374 in the fully bound, closed, and substrate-free, open states (Fig. S3). The dramatic increase in hydration of E374 in the absence of the substrate is expected to lower the pK_a value of E374, which may trigger release of the proton in the inward state. This scenario will be explored further in the next section.

We next consider the pK_a values of other titratable residues. The side chains of D368 and D455, which coordinate Na3 and Na1, respectively, give very large values of ΔΔG_pr, and consequently very low pK_a values. This is a direct consequence of the proximity of these two side chains to Na1 and Na3 and the absence of water molecules in this

FIGURE 5 (A) The region around E374 (purple) in the fully bound state, where this residue is buried under a series of hydrophobic residues from the HP2 and TM7 segments. (B) After the removal of the substrate and the Na2 ion, the HP2 gate opens and the E374 side chain is hydrated. To see this figure in color, go online.
region—there is only one water molecule in the binding sites of Na1, Na3 and the substrate (Fig. 4). Protonating a negatively charged aspartate side chain that coordinates a positively charged Na\(^+\) ion results in a very high energetic penalty, which is reflected in the calculated pK\(_a\) values. This indicates that the residues D368 and D455 have to be deprotonated to coordinate the Na1 and Na3 ions. We have shown previously that Na1 leaves the binding site shortly after D455 is protonated (36), which is consistent with the results obtained here. The D440 and D444 residues also give low pK\(_a\) values, most likely because they are also involved in charge interactions with the R447 side chain and the substrate \(\alpha\)-amino group, respectively (Fig. 4 A).

**pK\(_a\) values of E374 in different states of EAAT3**

In our previous study of EAAT3 (36), we obtained a series of states by running simulations with different combinations of bound ligands. In addition to the fully bound states in the outward- and inward-facing conformations, we also simulated these two conformations in the apo state and with only Na1 and Na3 bound to the transporter. Furthermore, we investigated the binding sites of the K\(^+\) ion in the inward model of EAAT3 and calculated the binding free energies of the K\(^+\) ion in three sites suggested by the mutagenesis experiments (36). Here, we calculate the pK\(_a\) value of E374 in all these states, as well as in the EAAT3 model built from the apo GltPh (Table 3). Our aim is to gain insights into the mechanism of proton binding and release and how that relates to the binding/unbinding of the other ligands. Because all our results are obtained using the EAAT3 model created from the fully bound GltPh, it is important to check its consistency by comparing the apo state result with that obtained from the EAAT3 model based on the apo GltTk template. As seen from the top two rows in Table 3, there is excellent agreement between the two results, confirming the relevance of the apo-state EAAT3 model used in the calculations. Comparison of the pK\(_a\) values between the outward and inward states for the apo, Na1-Na3 bound, and fully bound states (Table 3) shows that they are very similar. Even though this symmetry shows the consistency of our results, experiments based on the pH dependence of the forward and reverse transport modes reveal an asymmetry of 1.5 pK\(_a\) units between the outward- and inward-facing states of EAAT3 in the presence of the substrate (29). This discrepancy might arise from the free energies involved in the gating process of the substrate-bound EAAT3, which we do not consider in this study. This step isolates E374 from the solvent, directly affecting its pK\(_a\), and is likely to be different in the inward and outward states, as shown in the metadynamics studies of gate opening in GltPh (58).

We see from Table 3 that absence of the substrate and the Na2 ion (Na1, Na3 rows) has a great effect on the pK\(_a\) of this residue, which can be traced to hydration of E374 and the absence of the negatively charged substrate (Fig. 5). The pK\(_a\) value is reduced to 6.6 and 6.7 for the outward- and inward-states, meaning that around 85% of the E374 side chains are deprotonated when only Na1 and Na3 are bound. This shows that the binding of the substrate and the closure of the HP2 gate in the presence of the these two Na\(^+\) ions strongly favor proton binding to E374. To determine the relative contribution of these two processes, we have removed the Na2 ion from the fully bound outward state and simulated the system with a deprotonated E374 and a restrained substrate for 50 ns. In one of the chains of the transporter, we obtain a state in which Na1, Na3, and glutamate are bound, but the HP2 gate is fully open and E374 is hydrated. The pK\(_a\) value of E374 under these conditions is 10.4 (Table 3), which indicates that binding of glutamate is sufficient for the protonation of E374, and the closure of the gate further stabilizes it. As in GltPh, Na1 and Na3 are necessary for substrate binding to EAAT3, but these ions also favor the deprotonation of E374 and the consequent instability of the substrate in the binding site. On the other hand, the system with glutamate bound and a protonated E374 is very stable, and also produces a high value for the pK\(_a\) of this residue. Therefore, we propose that proton and substrate binding are mutually coupled, that is, substrate binding brings about the protonation of E374 and vice versa. This mechanism is corroborated by experiments, which show that in the pH range from 6 to 10, saturation of glutamate binding can be achieved by either increasing the glutamate concentration or decreasing the pH in the extracellular media (29). An interesting observation is that the pK\(_a\) value is 11.7 for both apo states, indicating a protonated E374 residue. We attribute this to the negative potential

<table>
<thead>
<tr>
<th>Ligands</th>
<th>ΔDG(_{ex})</th>
<th>ΔDG(_{back})</th>
<th>ΔDG(_{total})</th>
<th>ΔpK(_a)</th>
<th>pK(_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo</td>
<td>-10.4 ± 1.0</td>
<td>-10.1 ± 0.9</td>
<td>-10.2 ± 1.0</td>
<td>7.4 ± 0.7</td>
<td>11.7 ± 0.7</td>
</tr>
<tr>
<td>apo (GltTk)</td>
<td>-9.2 ± 1.5</td>
<td>-11.5 ± 1.2</td>
<td>-10.3 ± 1.4</td>
<td>7.5 ± 1.0</td>
<td>11.8 ± 1.0</td>
</tr>
<tr>
<td>Na1, Na3</td>
<td>-4.0 ± 0.9</td>
<td>-2.1 ± 1.1</td>
<td>-3.0 ± 1.0</td>
<td>2.2 ± 0.7</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>Na1, Na3</td>
<td>-8.9 ± 0.9</td>
<td>-7.7 ± 1.4</td>
<td>-8.3 ± 1.2</td>
<td>6.1 ± 0.9</td>
<td>10.4 ± 0.9</td>
</tr>
</tbody>
</table>

**TABLE 3** Calculated pK\(_a\) values of E374 with different ligands bound to the outward and inward states of EAAT3

<table>
<thead>
<tr>
<th>Ligands</th>
<th>ΔDG(_{ex})</th>
<th>ΔDG(_{back})</th>
<th>ΔDG(_{total})</th>
<th>ΔpK(_a)</th>
<th>pK(_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo</td>
<td>-10.2 ± 1.6</td>
<td>-10.1 ± 1.2</td>
<td>-10.2 ± 1.4</td>
<td>7.4 ± 1.0</td>
<td>11.7 ± 1.0</td>
</tr>
<tr>
<td>Na1, Na3</td>
<td>-3.3 ± 1.5</td>
<td>-3.4 ± 1.2</td>
<td>-3.3 ± 1.4</td>
<td>2.4 ± 1.0</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
<td>fully bound</td>
<td>-22.7 ± 1.1</td>
<td>-22.2 ± 0.9</td>
<td>-22.4 ± 1.0</td>
<td>16.3 ± 0.7</td>
<td>20.6 ± 0.7</td>
</tr>
<tr>
<td>K-site 1</td>
<td>-0.6 ± 1.0</td>
<td>-0.5 ± 1.1</td>
<td>-0.5 ± 1.0</td>
<td>0.4 ± 0.7</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>K-site 3</td>
<td>-4.0 ± 1.0</td>
<td>-3.3 ± 1.4</td>
<td>-3.6 ± 1.2</td>
<td>2.6 ± 0.9</td>
<td>6.9 ± 0.9</td>
</tr>
</tbody>
</table>

The forward and the backward calculations of the protonation free energies are listed in the second and third columns, respectively, and their average in the fourth column. The shift ΔpK\(_a\) is given in the fifth column, and the final value of the pK\(_a\) is given in the last column. Errors are estimated from block data analysis using 100 ps windows. All free energies are in kcal/mol.

*aWith the HP2 gate open and the hydrated E374 side-chain.*
induced by the negative charges of the D368 and the D455 residues, which are 10 and 15 Å away from E374, respectively. Even though there are water molecules between these residues and E374, they are mostly limited to the surface of the protein, and hence provide a limited dielectric screening compared to bulk water (Fig. 6 A). Presence of the Na1 and Na3 ions neutralizes these negative charges, thereby explaining the reduction in the pK_a value of E374 when the Na1 and Na3 ions are bound.

We next look at the release of the proton into the intracellular media, and how this is related to K^+ binding. Similar to the outward state, this proton is released when we remove the substrate and the Na2 ion, but it is bound if the transporter is empty because the pK_a of E374 is 11.7 in the inward-apo state (Table 3). To investigate the influence of K^+ binding on the proton release, we have also calculated the protonation state of E374 in the presence of K^+ in each of the three sites considered in our previous study (36). For K^+ site number 2, which is coordinated by the same E374 side chain, K^+ is not stable in the binding site and leaves it shortly after the protonation of this residue (not shown). Therefore, the deprotonation of E374 is absolutely necessary for K^+ binding at this site. In the case of K^+ sites 1 and 3, we obtain pK_a = 4.7 when K^+ is bound in site 1 and pK_a = 6.9 when K^+ is bound in site 3 (Table 3). This is a direct consequence of introduction of a K^+ ion in the apo state, which either replaces the Na1 and Na3 sodium ions (site 1) or the substrate α-amino group (site 3). Interestingly, introduction of one K^+ ion at site 1 causes a larger shift in the pK_a value compared to the apo state than the presence of both Na1 and Na3 (4.7 vs. 6.7). This can be explained because the binding of K^+ at site 1 not only reduces the negative potential in that region, but it also helps to hydrate the D368 side chain, which does not happen in the apo transporter (Fig. 6, A and B). In the case of site 3, the D444 side chain is part of the substrate binding site (coordinates its α-amino group), and is separated from E374 by the salt bridge between R447 and D440 (Fig. 6 C). The presence of a positive charge in this region, instead of the negatively charged substrate or an empty binding site, is responsible for reducing the pK_a value of E374, deprotonating this residue when K^+ is bound at site 3. These results show that binding of a K^+ ion is necessary for the deprotonation of the E374 side chain and the completion of the transport cycle.

CONCLUSIONS

In conclusion, we have performed rigorous pK_a calculations using the MD/FEP method to identify protonated residues in the human glutamate transporter EAAT3, and also to elucidate the mechanism of proton transport. Our calculations show that E374 is the only protonated residue in the fully bound outward-facing state, which agrees with experiments that point to this residue as the proton carrier in EAATs. We find that both the presence of the substrate and the closure of the HP2 gate contribute to the high pK_a value obtained in our calculations. A hydrophobic region formed by conserved residues in the TM7 and HP2 segments isolates E374 from the solvent, favoring the presence of a neutral side chain as opposed to a negatively charged one. When we remove the substrate and the Na2 ion, we see the opening of the HP2 gate and the hydration of the E374 side chain, which now has a pK_a value below the physiological pH and therefore should be mostly

FIGURE 6  (A) The region between E374 (purple) and the D368 and D455 side chains, which has water molecules located mostly on the surface of the protein, resulting in reduced dielectric screening. (B) The same area but now in the presence of the bound K^+ ion at site 1. We see that, in addition to the presence of the positively charged K^+ ion, the side chain of D368 is also hydrated. (C) The substrate binding site with a K^+ ion bound at site 3, which now has a positive charge instead of the negatively charged substrate, reducing the pK_a of E374. To see this figure in color, go online.
deprotonated. If we remove the remaining Na\(^+\) ions, the negative charges from the D368 and D455 side chains induce the protonation of E374, which now has a pK\(_a\) of \(\sim 12\). There is good agreement between the results from the apo states created from the fully bound GLT\(_{1b}\) and the apo GLT\(_{1b}\) templates, confirming the relevance of the former in apo state calculations.

To understand the mechanism of proton release, we perform the pK\(_a\) calculations in the inward-facing state of EAAT3 in the fully bound state, with only Na1 and Na3 bound, in the apo state, and also with K\(^+\) bound in three possible sites previously determined. There is good symmetry between the values in the inward and the outward conformations of EAAT3, with the inward apo state also having a protonated E374 carboxyl group. This result shows that the release of all the ligands and the hydration of E374 is not enough to deprotonate this residue, and therefore the proton release should depend on K\(^+\) binding to the inward transporter. Indeed, the binding of the K\(^+\) ion in any of the sites tested induces the deprotonation of E374, by either reducing its pK\(_a\) or directly coordinating this ion. This is consistent with a mechanism in which the K\(^+\) ion replaces either the Na1 and Na3 ions, the substrate, or the proton itself, so the transporter never needs to reach the inward apo state. This is in contrast to GLT\(_{1b}\), in which all the ligands have to be bound for the reorientation of the substrate to the outward state. This difference might explain the much faster transport rates of the human transporters compared to the archaeal ones, and also the need for the countertransport of a K\(^+\) ion in the EAATs.

**SUPPORTING MATERIAL**

Three figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00507-4.](http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00507-4.)

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**REFERENCES**

2. Reference deleted in proof.
3. Reference deleted in proof.