Conformational ensemble of the sodium-coupled aspartate transporter

Elka R Georgieva1,2, Peter P Borbat1,2, Christopher Ginter3, Jack H Freed1,2 & Olga Boudker3

Sodium and aspartate symporter from Pyrococcus horikoshii, GltPh, is a homolog of the mammalian glutamate transporters, homotrimeric integral membrane proteins that control neurotransmitter levels in brain synapses. These transporters function by alternating between outward-facing and inward-facing states, in which the substrate binding site is oriented toward the extracellular space and the cytoplasm, respectively. Here we used double electron-electron resonance (DEER) spectroscopy to probe the structure and the state distribution of the subunits in the trimer in distinct hydrophobic environments of detergent micelles and lipid bilayers. Our experiments reveal a conformational ensemble of protomers that sample the outward-facing and inward-facing states with nearly equal probabilities, indicative of comparable energies, and independently of each other. On average, the distributions varied only modestly in detergent and in bilayers, but in several mutants unique conformations were stabilized by the latter.

Glutamate is a major excitatory neurotransmitter in the central nervous system and is crucial in learning, memory formation and cognition. Although glutamate is abundant in the brain, its extracellular concentration is tightly controlled to allow for neurotransmission; abnormally elevated levels of glutamate observed in several neurodegenerative diseases, ischemia and epilepsy are toxic. Glutamate transporters are electrochemically driven pumps, which couple uptake of glutamate into the cytoplasm of astrocytes and neurons to the thermodynamically ‘downhill’ movements of ions. The structural information for this family comes from the crystal structures of an archaeal homolog, GltPh, a sodium and aspartate symporter7,10,11, which has ~35% amino acid sequence identity with the mammalian glutamate transporters and is a model system to study their mechanism. The crystal structures revealed a homotrimeric assembly, common for all characterized members of the family12,13. Each protomer has an independent set of aspartate and sodium (Na+) binding sites at the core of a peripherally located transport domain (Fig. 1a). The central transmembrane segments form the trimerization domain, mediating intersubunit contacts and remaining largely unchanged during transport8,9,14. In contrast, transport domains exhibit structural plasticity, leading to 15–18 Å movements across the membrane, thereby switching the substrate and ion-binding sites between the extracellular and cytoplasmic orientations, that is, the outward-facing and inward-facing states, respectively. Two reentrant hairpin segments of the transport domain, hairpins 1 and 2 (HP1 and HP2), occlude the substrate and ions from the aqueous milieus in both states, and HP2 has been proposed to serve as an extracellular gate7,15–17. Aspartate binding and closure of the extracellular gate are thermodynamically coupled to binding of Na+ ions. In contrast, isomerization between the outward-facing and inward-facing states has been proposed to occur independently of the ions, driven primarily by the thermal energy.

Here we aimed to gain structural information on outward-facing and inward-facing states of GltPh and to establish their energetic relationship in two distinct hydrophobic environments, namely in detergent micelles and in lipid bilayers. In this way we begin to sketch out the energy profile of the transport cycle. Toward this end, we used site-directed spin labeling and DEER spectroscopy to measure the distances between the paramagnetic probes. The technique provides access to distances and, notably, distance distributions ranging from 20 Å to over 80 Å, and has been applied to study diverse systems, including membrane proteins in lipid bilayers20,22–29. Here we broaden the applicability of DEER spectroscopy by extracting quantitative information on the populations and the energies of the conformational states contributing to the experimentally observed distance distributions.

We used spin-labeled cysteine mutants of GltPh for the long-range distance measurements either when the transporters were empty or loaded with Na+ ions and aspartate or a transport blocker dl-threo-β-benzoxyaspartate (TBOA). We obtained broad distance distributions, which we interpret in terms of an ensemble of the underlying inward-facing and outward-facing states, consistent with the known crystal structures. The quantitative analysis revealed that the two states were populated with almost equal probabilities under all conditions, indicating similar energies. Moreover, our data are consistent with a lack of cooperativity in the trimers, painting a picture of a system in which each protomer samples outward-facing and inward-facing conformations independently of its neighbors, regardless of whether it is loaded with the substrate or is empty. On average, we found no

1National Biomedical Center for Advanced Electron Spin Resonance Technology, Cornell University, Ithaca, New York, USA. 2Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York, USA. 3Weill Cornell Medical College, Department of Physiology and Biophysics, New York, New York, USA. Correspondence should be addressed to O.B. (olb2003@med.cornell.edu).

Received 14 June 2012; accepted 17 December 2012; published online 20 January 2013; doi:10.1038/nsmb.2494
substantial differences in either the structure or the relative energies of the outward-facing and inward-facing states in detergent micelles and in lipid bilayers. However, in several mutants, in which the spin label was placed on the interface between the transport and trimerization domains in one of the states, we saw substantial stabilization of those states in lipid but not in detergent. These results suggest that the spin label participates in specific protein interactions and that these interactions are more favorable when the transporter is embedded into membrane compared to detergent micelles.

RESULTS

Spin-labeling strategy

Isomerization of GltPb between the outward-facing and inward-facing states as seen by crystallography6–8 involves ~15 Å translation and 35° rotation of the transport domains relative to the trimerization domain (Fig. 1a). As a result, the intersubunit distances between equivalent residues in the transport domains change by up to 20 Å, providing a means to distinguish the states. For single-cysteine mutagenesis and labeling, we selected residues that are solvent-accessible in at least one of the conformations and for which the intersubunit distances are ~20–70 Å, which are most suitable for DEER analysis (Fig. 1b, and Supplementary Table 1). Specifically, we chose two regions distant from the substrate-binding and ion-binding sites, one on each the cytoplasmic and the extracellular side, and the third region on HP2 (Fig. 1c). Two residues in the trimerization domain, which is expected to remain rigid upon substrate binding or translocation, served as controls. We spin-labeled the purified cysteine mutants and conducted DEER measurements (Supplementary Fig. 1 and Supplementary Note) either in detergent (n-dodecyl-β-D-maltopyranoside; DDM) or after reconstitution into lipid bilayers (1-palmitoyl-2-oleoylphosphatidylcholine; POPC).

Structural stability of the trimerization domain

For the labeled trimerization domain mutant, K55R1 (R1 denotes a spin-labeled cysteine), the amplitude of the DEER signal at zero time in detergent was consistent with complete spin labeling of cysteines (Fig. 2a, Supplementary Fig. 2a and Supplementary Note). The clear oscillations in the time evolution signal indicated a narrow range of the interspin distances. The extracted distance distributions covered a range between 18 Å and 30 Å for the substrate-free apo GltPb and the transporter bound to Na+ and aspartate or to TBOA. We obtained similar results in detergent and lipid. The distribution medians (Fig. 2b) varied between 23 Å and 28 Å, in excellent agreement with the Cβ-Cβ interprotomer distances in the crystal structures of the outward-facing and inward-facing GltPb (Fig. 1b and Supplementary Table 1). The distribution widths (2.8–4.6 Å) were similar to those obtained for soluble well-folded proteins30–32 and likely originated mainly from the conformational flexibility of the R1 side chain.

We obtained similar but distinct signals for GltPb, K55R in the apo state and when bound to either aspartate or TBOA, particularly in detergent. Residue 55 is at the interface of the transport and trimerization domains, and likely experiences distinct steric constraints in the outward-facing and inward-facing states, which may underlie differences in the interspin distances. To test this, we computed theoretical distance distributions based on energy-weighted R1 rotamer libraries generated by a modeling program MMM13 using crystallographic structures of GltPb in the symmetric outward-facing and inward-facing states along with a model of an asymmetric trimer with two protomers in the outward-facing state and one in the inward-facing state (Online Methods). Modeling suggested that R1 in position 55 was more sterically constrained in the inward-facing conformation with fewer accessible rotamers (Fig. 2c and Supplementary Fig. 2b). Notably, experimental distance distributions encompassed predicted features of both the outward-facing and inward-facing states, suggesting that both were populated. We obtained similar results for V216R1 mutant, located in a more flexible peripheral part of the trimerization domain. Consistently, the experimental distance distributions were broader with widths of 5.2–7.6 Å (Supplementary Fig. 3 and Supplementary Table 1).

To evaluate potential contributions from the three-spin effects to the apparent distance distributions34,35, we collected DEER data...
on Glt\textsubscript{p5}-K55R1 using pump pulses with decreasing amplitude (Supplementary Note).

As expected for a three-spin system, strong pulses of 13 gauss (G) yielded an extra peak around ~23 Å, which was diminished when we used weaker pulses that flipped only a fraction of the spins (Fig. 2d). Therefore, in subsequent experiments we used a 5.6-G pump pulse, which rendered the three-spin effects negligible.

**Dynamic nature of the transport domain**

We collected DEER data on eight transport domain mutants in detergent and lipid membranes either in the apo state or bound to Na\textsuperscript{+} and aspartate or to TBOA, yielding 44 different samples. We obtained very broad distance distributions, spanning up to 40–50 Å, for almost all samples except in a few cases (Fig. 3 and Supplementary Fig. 4). In most of the detergent-containing samples, additions of Na\textsuperscript{+} and aspartate or Na\textsuperscript{+} and TBOA shifted the distance-distribution midpoints toward longer distances in the extracellular mutants (by an average of 2.2 Å or 4.3 Å, respectively) and toward shorter distances in the intracellular mutants (by ~2.8 Å or 3 Å, respectively). These systematic changes suggest that the broad distance distributions are not due to local flexibility but arise from Glt\textsubscript{p5} protomers populating independently both the outward-facing and inward-facing states, and that binding of both aspartate and TBOA in detergent favors the outward-facing state to some extent. It is, in principle, possible that the distance distributions are due to the protomers sampling not only the fully outward-facing and inward-facing states but the intermediate positions as well. Indeed, a recent crystal structure has captured a Glt\textsubscript{p5} protomer in an intermediate orientation\textsuperscript{9}, suggesting that the energies of the intermediate states may not be prohibitively high. However, we think that the intermediates do not contribute to the conformational ensemble because the distributions obtained in the presence of TBOA are similarly broad. The benzyl group of TBOA is expected to prevent the occlusion of the inhibitor in the binding site, disabling translocation\textsuperscript{7,36}. Therefore, the broad distributions must originate from the contributions of Glt\textsubscript{p5} protomer pairs in discrete configurations. Modeling also showed that the experimental distance distributions could not be explained by a single conformation because they covered the distances predicted for both states (Supplementary Fig. 4). These observations suggest that in detergent solutions and in lipid bilayers, both in the bound and unbound states, Glt\textsubscript{p5} protomers are distributed between the outward-facing and inward-facing conformations.

**Conformational selection in the lipid bilayer**

Overall, distance distributions were similarly broad in detergent micelles and in lipid bilayers. However, there were several exceptions, including K290R1 bound to TBOA, E296R1 in either apo or bound states and A364R1 bound to aspartate, for which the distributions were broad in detergent but narrow in the bilayers. Particularly for residue 296, we observed very narrow peaks in the distance distributions, indicative of steric constraints on R1 mobility. The distribution medians in the bilayers were consistent with the outward orientation of the intracellular mutants K290R1 and E296R1, and inward orientation of the extracellular mutant A364R1. The crystal structures suggest that these residues are not in a direct contact with the lipid in either the outward-facing or inward-facing states. If this is the case, why do the R1 side chains stabilize specific states in lipid bilayers and not in detergents? The intracellular residues 290 and 296 are located on the interface between the transport and trimerization domains in the outward-facing state, where they are well positioned to participate in the interdomain interactions. In contrast, they are distant from the interface in the inward-facing state. The opposite is true for residue 364, which is adjacent to the trimerization-domain Lys55 in the inward-facing state but not outward-facing state. Hence, we hypothesize that the nitroxide side chains, which have both hydrophobic character and hydrogen-bond acceptor potential, are engaged in the interdomain interactions and that the favorable energetic contributions of such interactions are larger in the context of the lipid bilayer compared to detergent micelles. For residue 364, we observed stabilization of the inward-facing state in membranes only in complex with aspartate but not TBOA. Consistently, in the crystal structure of the TBOA-bound Glt\textsubscript{p5}, HP2 is in an open conformation, and residue 364 is distant from the domain interface.

**Quantitative analysis of the distance distributions**

Because the broad experimental distributions suggested that both outward-facing and inward-facing states were present, we aimed to determine their populations. Computational predictions based on R1 rotamer libraries yielded average distances that agreed well with the experiment (Supplementary Table 1), but the predicted and experimental distance distributions differed substantially. Hence for quantitative analysis, we modeled the experimental data as sums of three Gaussian functions, representing the distance distributions...
originating from possible configurations of the subunit pairs: both outward, both inward or mixed, that is, one outward and one inward. Assuming that the protomers isomerize between outward-facing and inward-facing states independently of each other, and taking the probability of a protomer to be in the outward-facing state as $P_O$, the probabilities of the two neighboring subunits facing outward or inward are $P_O^2$ and $(1-P_O)^2$, respectively, and the probability that they are in the mixed configurations is $2P_O(1-P_O)$. In mixed configurations, the distances from outward-facing to inward-facing and from inward-facing to outward-facing protomers are not quite the same, as the transport domain movements combine translation and rotation. Nevertheless, we represented them as a single Gaussian because increasing the number of the optimized parameters was statistically unjustified. The distance probability, $P(r)$, is then:

$$P(r) = S \times \left( \frac{P_O}{\sigma_O} G_O + \frac{2P_O(1-P_O)}{\sigma_M} G_M + \frac{(1-P_O)^2}{\sigma_I} G_I \right)$$

where $G_O$, $G_M$, $G_I$ and $\sigma_O$, $\sigma_M$ and $\sigma_I$ are the Gaussian means ($G$) and s.d. ($\sigma$) for the outward-facing, mixed and inward configurations, respectively; and $S$ is a normalization factor. The areas of the Gaussians are proportional to configuration probabilities. As initial guesses for the Gaussian means, we used either $C_{\beta}$-$C_{\beta}$ distances or distances derived from modeling, which we then optimized locally in the parameter space. During the fitting process, we generally constrained the Gaussian widths to less than 6 Å, an approximate upper limit for the distance distributions measured for structured residues.$^{30-32}$ The majority of the Gaussians satisfied this criterion, with a few exceptions when broader widths had to be used to adequately represent the distributions (Supplementary Table 1). The resulting fits described well both the distance distributions (Fig. 3) and the time-domain raw data (Supplementary Fig. 5a). To verify the robustness of the data analysis and fitting, we varied the baseline, subtracted from the time-domain data, which gave negligible variations (Supplementary Fig. 6b, Supplementary Table 2 and Supplementary Note).

Distances are consistent with the crystal structures

From the Gaussian fits, we extracted 139 distances, which were overall in excellent agreement with the $C_{\beta}$-$C_{\beta}$ distances and with distances calculated from the rotamer libraries (Fig. 4a and Supplementary Table 1), yielding r.m.s. deviation of ~6 Å. Notably, 20 distance measurements between residues in T4 lysozyme$^{31}$ yield r.m.s. deviation of 6.9 Å, suggesting that these values reflect the inherent differences between the $C_{\beta}$-$C_{\beta}$ distances and those between the paramagnetic atoms of R1. The r.m.s. deviations calculated for the subsets of distances attributed to the symmetric outward-facing and inward-facing conformations (Fig. 4b). We also obtained similar r.m.s. deviations for measurements conducted in lipids and in detergent. However on average, distances in lipid bilayers were shorter than in detergent, suggesting that the overall protein structure was more compact (Fig. 4c). Finally, r.m.s. deviations for the apo, aspartate-bound and TBOA-bound GltPh mutants in detergent were 6.2 Å, 5.9 Å and 5.9 Å, respectively, and in lipid, 5.6 Å, 4.7 Å and 6.3 Å, respectively (Fig. 4d), suggesting that no structural rearrangements involving the labeled residues took place that would be incompatible with the crystal structures.

The Gaussian means for the symmetric outward-facing and inward-facing conformations are scattered about the crystallographic $C_{\beta}$-$C_{\beta}$ distances with a small bias toward longer distances (Fig. 4a-d). Such a bias is typically observed for globular proteins, in which the R1 side chains are more likely to point away from the protein and from each other$^{37}$. We also detected several outliers, for which the predicted and measured distances differed by as much as 8–15 Å (Supplementary Table 1). In three cases (residues 294, 364 and 378 in the inward-facing
The states have close energies

Fitted values of the outward-facing state probabilities varied between mutants. However, on average, they were 0.4–0.8 for all ligand conditions and hydrophobic environments (Fig. 5a), suggesting that the outward-facing and inward-facing states were similarly populated and hence, were similar in energy. We found that three Gaussian functions with areas weighted by the binomial coefficients described the data well, consistent with the individual protomers in the Gltpb trimer sampling conformational states independently of each other. Although our measurements cannot exclude subtle coupling between the subunits, we note that all-or-none transitions, such that the subunits are either all outward-facing or inward-facing, are neither required nor prohibited. Instead, we observed a range of distributions. In some, the symmetrical states were dominant, but in others the mixed states were substantially populated. These results are in agreement with the previous functional experiments, postulating independence of the subunits in Gltpb and the mammalian glutamate transporters. On average, aspartate, and more so TBOA binding, modestly shifted the distributions toward the outward-facing state. These results are consistent with our recent measurements showing that the outward-facing and inward-facing states have similar affinities for Asp, and that the former binds TBOA better, consistent with the individual protomers in the Gltpb trimer sampled conformations similarly.

Figure 5 The inward and outward-facing states of Gltpb have close energies. (a) Average populations of the outward-facing state for the indicated ligand conditions (error bars, s.e.m.; n = 8), obtained from the Gaussian fits of data collected in detergent (DDM) and in lipid (POPC).
that shifting the energy balance between the outward-facing and inward-facing states did not abrogate transport.

**DISCUSSION**

In membrane transporters, transitions between the outward-facing and inward-facing states are the linchpin of the catalytic mechanism. These isomerizations occur both in the substrate-loaded and substrate-free transporters to complete the cycle (Fig. 5b). Crystal structures of the wild-type GltPh have pictured the protein in symmetric outward-facing states. The structure of the symmetric inward-facing state had been determined by trapping the transporter using mercury-mediated crosslinking of two cysteines placed on the domain interface and later recapitulated in the absence of the crosslinks. These structures pictured the outward-to-inward transition involving a large-scale transmembrane movement of the transport domain. Here we demonstrated that these domain motions occur in detergent solution and, importantly, in membranes. We also developed a more complete picture of the conformational ensemble of the transporter by showing that the outward-facing and inward-facing states had similar energies both when the transporter was fully loaded with Na+ and aspartate and empty. Our measurements were consistent with the lack of either positive or negative cooperativity between the subunits of the trimer, picturing GltPh as a system of three largely independent protomers sampling the outward-facing and inward-facing states. GltPh originates from a hyper-thermophilic archaeon and normally protomers sampling the outward-facing and inward-facing states.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGMENTS**

The work was supported by US National Institute of Neurological Disorders and Stroke grants NS064357 and NS064357-02S1 and a grant from Bohmcharitable Trust to O.B., and US National Center for Research Recourses grant P41-RR016292, US National Institute of General Medical Sciences grant P41GM103521 and US National Institute of Biomedical Imaging and Bioengineering grant R01EB003150 J.H.F.

**AUTHOR CONTRIBUTIONS**

E.R.G. and O.B. developed the mutation strategy for DEER spectroscopy, and designed the molecular biology and biochemical part of the experiments. E.R.G., O.B. and P.P.B. analyzed the data. O.B., E.R.G., P.P.B. and J.H.F. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nsmb.2494. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Danbolt, N.C. Glutamate uptake. *Prog. Neurobiol.* **65**, 1–105 (2001).
2. Hinoi, E., Takarada, T., Tsuchihashi, Y. & Yoneda, Y. Glutamate transporters as drug targets. *Curr. Drug Targets CNS Neurol. Disord.* **4**, 211–220 (2005).
3. Levy, L.M., Wann, O. & Attwell, D. Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a Chinese hamster ovary cell line selected for low endogenous Na+-dependent glutamate uptake. *J. Neurosci.* **18**, 9620–9628 (1998).
4. Zerangue, N. & Kavanaugh, M.P. Flux coupling in a neuronal glutamate transporter. Nature 383, 634–637 (1996).

5. Owe, S.G., Maraggi, P. & Attwell, D. The ionic stoichiometry of the GLAST glutamate transporter in salamander retinal glia. J. Physiol. (Lond.) 577, 591–599 (2006).

6. Yernool, D., Boudker, O., Jin, Y. & Gouaux, E. Structure of a glutamate transporter homolog in the cyanobacterium Synechocystis. Nature 431, 811–818 (2004).

7. Boudker, O., Ryan, R.M., Yernool, D., Shimamoto, K. & Gouaux, E. Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter. Nature 445, 387–393 (2007).

8. Reyes, N., Ginter, C. & Boudker, O. Transport mechanism of a bacterial homologue of glutamate transporters. Nature 462, 880–885 (2009).

9. Verdon, G. & Boudker, O. Crystal structure of an asymmetrical trimer of a bacterial glutamate transporter homolog. Nat. Struct. Mol. Biol. 19, 355–357 (2012).

10. Ryan, R.M., Compton, E.L. & Mindelli, J.A. Functional characterization of a sodium-dependent aspartate transporter from Pyrococcus horikoshii. J. Biol. Chem. 284, 17540–17548 (2009).

11. Groeneveld, M. & Slotboom, D.J. Na+: aspartate coupling stoichiometry in the glutamate transporter homologue GLP. Biochemistry 49, 3511–3513 (2010).

12. Yernool, D., Boudker, O., Folta-Stogniew, E. & Gouaux, E. Trimeric subunit stoichiometry of the glutamate transporters from Bacillus caldotenax and Bacillus steaerothermophilus. Biochemistry 42, 12981–12988 (2003).

13. Gendreau, S. et al. A trimeric quaternary structure is conserved in bacterial and human glutamate transporters. J. Biol. Chem. 279, 39505–39512 (2004).

14. Groeneveld, M. & Slotboom, D.J. Rigidity of the subunit interfaces of the trimeric glutamate transporter GltT during translocation. J. Mol. Biol. 372, 565–570 (2007).

15. Focke, P.J., Moenn-Loccoz, P. & Larsson, H.P. Opposite movement of the external gate of a glutamate transporter homolog upon binding cotransported sodium compared with substrate. J. Neurosci. 31, 6259–6262 (2011).

16. Huang, Z. & Tajkhorshid, E. Dynamics of the extracellular gate and ion-substrate coupling in the glutamate transporter. Biophys. J. 95, 2292–2300 (2008).

17. Shrivastava, I.H., Jiang, J., Amara, S.G. & Bahar, I. Time-resolved mechanism of extracellular gate opening and substrate binding in a glutamate transporter. J. Biol. Chem. 283, 28680–28690 (2008).

18. Borbat, P.P. & Freed, J.H. Measuring distances by pulsed dipolar ESR spectroscopy: spin-labeled histidine kinases. Methods Enzymol. 423, 52–116 (2011).

19. Borbat, P.P. & Freed, J.H. Pros and cons of pulse dipolar ESR: DQ and DEER. EPR Newsletter 17, 21–33 (2011).

20. Mchaourab, H.S., Steed, P.R. & Kazmier, K. Toward the fourth dimension of optimized EPR distance restraints for protein structure determination. J. Struct. Biol. 173, 549–557 (2011).

21. Georgieva, E.R. et al. Effect of freezing conditions on distances and their distributions derived from double electron electron resonance (DEER): a study of doubly-spin-labeled T4 lysozyme. J. Magn. Reson. 216, 69–77 (2012).

22. Zhang, Z., Boudker, O., Bort, P.P., Wang, J., Freed, J.H. & Edmondson, D.E. Determination of the oligomeric states of human and rat monoamine oxidases in the outer mitochondrial membrane and octyl beta-D-glucopyranoside micelles using pulsed dipolar electron spin resonance spectroscopy. Biochemistry 47, 1554–1566 (2008).

23. Vamvouka, M., Cieslak, J., Van Eps, N., Hubbell, W. & Gross, A. The structure of the lipid-embedded potassium channel voltage sensor determined by double-electron-electron resonance spectroscopy. Protein Sci. 17, 506–517 (2008).

24. Clixton, D.P. et al. Ion/substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter/sodium symporters. Nat. Struct. Mol. Biol. 17, 822–829 (2010).

25. Deyo, B., Jeschke, G., Goetz, B.A., Locher, K.P. & Bordignon, E. Transmembrane gate movements in the type II ATP-binding cassette (ABC) importer BtuCDF during nucleotide cycle. J. Biol. Chem. 286, 41008–41017 (2011).

26. Borbat, P.P., Mchaourab, H.S. & Freed, J.H. Protein structure determination using long-distance constraints from double-quantum coherence ESR: study of T4 lysozyme. J. Am. Chem. Soc. 124, 5304–5314 (2002).

27. Kazmier, K., Alexander, N.S., Meiler, J. & Mchaourab, H.S. Algorithm for selection of optimized EPR distance restraints for de novo protein structure determination. J. Struct. Biol. 173, 549–557 (2011).

28. Georgeria, E.R. et al. Full-length structure of the fourth domain of the sodium-hydantoin transporter Mhp1. Proc. Natl. Acad. Sci. USA 104, 18029–18030 (2007).

29. Phillips, R., Ursell, T., Wiggins, P. & Sens, P. Emerging roles for lipids in shaping membrane-protein function. Nature 459, 379–385 (2009).

30. Andersen, O.S. & Koppel, R.E., II. Bilayer thickness and membrane protein function: an energetic perspective. Annu. Rev. Biophys. Biomol. Struct. 36, 107–130 (2007).

31. Lee, A.G. How lipids affect the activities of integral membrane proteins. Biochim. Biophys. Acta 1656, 62–87 (2004).

32. Van Dam, J.T., Jaszewski, T.M., Fleming, K.G. & MacKenzie, K.R. Changes in apparent free energy of helix-helix dimerization in a biological membrane due to point mutations. J. Mol. Biol. 371, 422–434 (2007).

33. Snider, V., et al. Site-directed alkylation and the alternating access model for periplasmic permeation pathways in individual neuronal glutamate transporter subunits. J. Neurosci. 27, 2938–2942 (2007).

34. Zhang, Z. et al. Transport direction determines the kinetics of substrate transport by the glutamate transporter EAAC1. Proc. Natl. Acad. Sci. USA 104, 18029–18030 (2007).

35. Phillips, R., Ursell, T., Wiggins, P. & Sens, P. Emerging roles for lipids in shaping membrane-protein function. Nature 459, 379–385 (2009).

36. Andersen, O.S. & Koppel, R.E., II. Bilayer thickness and membrane protein function: an energetic perspective. Annu. Rev. Biophys. Biomol. Struct. 36, 107–130 (2007).

37. Lee, A.G. How lipids affect the activities of integral membrane proteins. Biochim. Biophys. Acta 1656, 62–87 (2004).

38. Van Dam, J.T., Jaszewski, T.M., Fleming, K.G. & MacKenzie, K.R. Changes in apparent free energy of helix-helix dimerization in a biological membrane due to point mutations. J. Mol. Biol. 371, 422–434 (2007).

39. Kaback, H.R. et al. Site-directed alkylation and the alternating access model for LacY. Proc. Natl. Acad. Sci. USA 104, 491–494 (2007).

40. Shimamura, T. et al. Molecular basis of alternating access membrane transport by the sodium-hydrion transporter Mhp1. Science 328, 470–473 (2010).
ONLINE METHODS

Molecular biology and protein purification. Mutations encoding cysteine substitutions were introduced in the sequence encoding cysteine-less seven-histidine GltPh variant using standard techniques and verified by DNA sequencing. All mutants were expressed and purified as described previously. Briefly, C-terminal (His)_8 fusion constructs were expressed in *Escherichia coli* DH10B cells (Invitrogen) and purified by metal affinity chromatography. The tag was removed by thrombin digestion, and the proteins were further purified by size-exclusion chromatography. The final protein concentration of trimers was 15–30 μM, calculated from absorbance at 280 nm using coefficient of 26, 820 M⁻¹ cm⁻¹ (per monomer). Samples were stored at −80 °C and further manipulated in buffer A containing in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 mM aspartate and 1 mM DDM.

Spin-labeling and preparation of samples for DEER spectroscopy. All mutants were spin-labeled with S-(2,5,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate (MTSL; Toronto Research Chemicals) in buffer A. MTSL was first dissolved in acetonitrile and added to the protein solutions in 20–30-fold (per monomer) molar excess. The labeling was performed for 3 h at room temperature and continued overnight at 4 °C with agitation. The unreacted label was removed by dialysis against four changes of 30 ml of buffer A over 30 h using 25-kDa molecular weight cutoff membrane. To prepare substrate-free GltPh, buffer B, containing 20 mM HEPES, pH 7.4, 100 mM KCl and 1 mM DDM, was used during the last two buffer changes. When apo protein samples were prepared for reconstitution into the bilayers, buffer B was supplemented with 2.5 mM NaCl. TBOA-bound protein was prepared starting with apo protein by adding NaCl and TBOA (Tocris Bioscience) to 50 mM and 125 μM, respectively. The liposome suspension was filtered, supplemented with fresh beads and incubated for 4 h at 4 °C. The procedure was repeated twice. The multilayer vesicles were collected by centrifugation at 70,000g for 15 min. The vesicles containing apo GltPh were additionally washed three times by loading with DDM-free buffer B using freeze and thaw procedure followed by centrifugation. To prepare TBOA-bound lipid-reconstituted samples, the vesicles were washed in the same manner in buffer supplemented with 200 mM NaCl and TBOA at 50 mM and 125 μM, respectively. The final concentration of the spin-labeled GltPh trimer in both DDM and POPC samples was 10–55 μM, based on the standard calibrated measurement of the primary echo amplitude, which gave total spin concentration. The reference sample was 200 μM 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) in 50% (w/v) glycerol-d₈/D₂O₂5. The final GltPh trimer to lipid molar ratio was 1:2,400, and lipid concentration was ~75 mg/ml. All samples were prepared in the final buffer containing 90% D₂O with protein samples in DDM also supplemented with glycerol-d₈ at 20% (w/v). Samples were loaded into 1.8 inner diameter custom Pyrex sample tubes (Wilnald-LabGlass), and shock-frozen in liquid N₂ before DEER measurements. Samples were prepared and measured at least twice.

DEER measurements. Measurements were performed at 60 K as previously described using a 17.3 GHz home-built pulse spectrometer, which has provided high-sensitivity measurements in previous studies. A standard four-pulse DEER sequence with π/2−π−π−π/2 pulse widths of 16 ns, 32 ns and 32 ns, respectively, and a 32 ns π pump pulse was used routinely. When testing the three-spin effects, a 14-ns pump pulse with varied flip angles was used (Supplementary Note). The frequency separation between detection and pump pulses was 70 MHz. The detection pulses were positioned at the low-field edge of the nitroxide spectrum. Typical dipolar evolutions times were 1.5–5 μs as needed with signal averaging from 2 h to 20 h. The homogeneous background was removed from the raw time-domain signals and the distances were reconstructed from the baseline-corrected and normalized signals by using Tikhonov regularization method and refined by maximum entropy method (Supplementary Note).

Modeling of distance distributions and data analysis. To model expected distance distributions, we used rotamer libraries generated by molecular modeling software MM3. The ensembles of statistically weighed spin-label conformers were calculated at 175 K. The crystal structures of GltPh in the outward-facing conformation with substrate (PDB: 2NWX) or TBOA (PDB: 2NWV) and inward-facing conformation with substrate (PDB: 3KBC) were used as templates for the calculations. In addition, we generated a structural model containing two protomers in the outward-facing and one protomer in the inward-facing conformation. The model construction was straightforward because the conformations of the trimerization domains are similar in the outward-facing and inward-facing states, and there are no steric clashes. For the mixed states, we calculated the average distance distributions from outward-facing to inward-facing and from inward-facing to outward-facing protomers. The experimental distance distributions were fitted to a single Gaussian for residues in the trimerization domain and to a scaled sum of three Gaussians for residues in the transport domain using nonlinear curve fitting algorithm in OriginLab software (OriginLab). As initial guesses for the Gaussians means, we used the average distances calculated by MM3, or the C₈-C₉ distances in the crystal structures and the structural model.

Activity assay. For the transport activity measurements, spin-labeled and unlabeled GltPh mutants were reconstituted in POPC liposomes at protein to lipid ratio of 1:100 as previously described. Porteoliposomes were loaded with buffer containing 20 mM HEPES, pH 7.4, 100 mM KCl and 200 mM choline chloride, extruded through 0.4 µm nitrocellulose filters and incubated for 2 min at 30 °C in buffer containing 20 mM HEPES, pH 7.4, 100 mM KCl and 200 mM NaCl and 600 mM [3H]l-aspartate. The uptake reactions were terminated by diluting porteoliposomes into ice-cold buffer, vesicles were collected by filtration, and retained radioactivity was measured.

51. Borbat, P.P., Crepeau, R.H. & Freed, J.H. Multifrequency two-dimensional Fourier transform ESR: an X/Ku-band spectrometer. *J. Magn. Reson.* **Reson.** 127, 155–167 (1997).
52. Borbat, P. & Freed, J.H. Pulse dipolar ESR: distance measurements. In *Structural Information from Spin-Labels and Intrinsic Paramagnetic Centres in the Biosciences*. *Structure and Bonding.* (eds., Harmer, J. & Timmel, C.) (Springer, Berlin, 2012).
53. Pannier, M., Veit, S., Godt, A., Jeschke, G. & Spiess, H.W. Dead-time free measurement of dipole-dipole interactions between electron spins. *J. Magn. Reson.* **Reson.** 142, 331–340 (2000).
54. Chiang, Y.W., Borbat, P.P. & Freed, J.H. The determination of pair distance distributions by pulsed ESR using Tikhonov regularization. *J. Magn. Reson.* **Reson.** 172, 279–295 (2005).
55. Chiang, Y.W., Borbat, P.P. & Freed, J.H. Maximum entropy: a complement to Tikhonov regularization for determination of pair distance distributions by pulsed ESR. *J. Magn. Reson.* **Reson.** 177, 184–196 (2005).