Binding thermodynamics of a glutamate transporter homolog

Nicolas Reyes1,2, SeCheol Oh1 & Olga Boudker1

Glutamate transporters catalyze concentrative uptake of the neurotransmitter into glial cells and neurons. Their transport cycle involves binding and release of the substrate on the extracellular and intracellular sides of the plasma membranes and translocation of the substrate-binding site across the lipid bilayers. The energy of the ionic gradients, mainly sodium, fuels the cycle. Here, we used a cross-linking approach to trap a glutamate transporter homolog from Pyrococcus horikoshii in key conformational states with the substrate-binding site facing either the extracellular or the intracellular side of the membrane to study binding thermodynamics. We show that the chemical potential of sodium ions in solution is exclusively coupled to substrate binding and release, not to substrate translocation. Despite the transporter’s structural symmetry, the binding mechanisms are distinct on the opposite sides of the membrane and more complex than the current models suggest.

Ion-coupled active transporters move their substrates against concentration gradients by using the energy of ionic gradients. To achieve this, the coupled ions drive cycles of conformational changes, which entail the transporter binding its substrate on one side of the membrane, translocating and releasing it on the other side and finally returning to the initial conformation (Fig. 1a). Despite the wealth of the functional and structural data supporting this view, the role of ions in this process remains incompletely understood, and several mechanisms of coupling have been proposed1. Some of them suggest that the ions control the substrate-binding affinity on the opposite sides of the membrane, whereas others focus on their role in modulating the kinetics of the translocation steps1,2.

The paucity of the confirmed coupling mechanisms is largely due to the experimental challenges of dissecting the role of ions in substrate binding and in translocation reactions. To circumvent these difficulties and to probe the role of ions in the isolated steps of a transport cycle, we have developed a new experimental approach, using a prokaryotic homolog of the glutamate transporter family, GltPβ, as a model system. GltPβ originates from a hyperthermophilic archaean, P. horikoshii, and symports aspartate and sodium with a 1:3 substrate/ion stoichiometry3,4. It is the only member of the family with known high-resolution structures5–8. The crystallographic studies have shown that the translocation of the substrate-binding site across the membrane involves a 15- to 18-Å transmembrane movement of a peripheral transport domain relative to a central trimerization domain (Fig. 1b)5–8. The latter mediates the intersubunit contacts within the trimeric GltPβ, whereas the former coordinates Na+ ions and substrate l-aspartate. The aspartate- and two Na+–binding sites have been identified crystallographically; the location of the third Na+ site is still debated6,9–15. The first Na+ site (Na1) is located deep in the core of the transport domain, whereas the second (Na2) is on the surface and is formed in part by the helical hairpin 2 (HP2; Fig. 1b). Notably, bound aspartate does not contribute directly to either of the sites. The structures of the unbound transporter, and hence the mechanisms by which the substrate and ions are released, remain unknown. However, a clue is provided by a structure of GltPβ in the outward-facing state in complex with a competitive blocker, l-threo-β-benzylxaspartate (l-TBOA)6, in which the blocker is bound in the substrate-binding pocket with its benzyl group propping HP2 in an open conformation and disrupting the Na2 binding site, which suggests that HP2 serves as the extracellular gate of the transporter6,16–18.

To dissect the mechanism of the energetic coupling between binding of Na+ ions and aspartate to the isolated outward and inward-facing states of GltPβ, we introduced pairs of cysteine mutations, one in the transport domain and the other in the trimerization domain, which upon cross-linking trapped the transporter in either of these states and disallowed the translocation steps (Fig. 1b). Using this approach, we show that, in both the outward- and inward-facing states, binding of aspartate is coupled to binding of all three cotransported Na+ ions and that binding of the ligands is highly cooperative. Hence, essentially all available chemical potential of Na+ ions is coupled to substrate binding on the opposite sides of the membrane, whereas other conformational changes, including the transmembrane movements of the transport domains, occur independently of the ions. Furthermore, on the basis of experimentally determined heat-capacity changes upon binding, we conclude that despite similar structure of the substrate- and ion-binding sites in the outward- and inward-facing states, the molecular events underlying binding, particularly of the first two Na+ ions, are distinct in these states and are more complex than available crystal structures would suggest.

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1Department of Physiology and Biophysics, Weill Cornell Medical College, New York, New York, USA. 2Present address: Centre National de la Recherche Scientifique, Unités Mixtes de Recherche 3528, Molecular Mechanisms of Membrane Transport Unit, Department of Structural Biology and Chemistry, Institut Pasteur, Paris, France. Correspondence should be addressed to O.B. (olb2003@med.cornell.edu) or N.R. (nicolas.reyes@pasteur.fr).

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RESULTS

Trapping GlpPh in the outward- and inward-facing states

To characterize the binding properties of the main conformational states of GlpPh, we ‘stapled’ together the transport and trimerization domains in either the outward- or inward-facing states by using Hg\(^{2+}\)-mediated cross-linking of double-cysteine mutants. Previous studies showed that in Hg\(^{2+}\)-cross-linked GlpPh K55C A364C (GlpPh\(^{\text{in}}\)), the transport domain is trapped in the inward-facing position\(^7\). By analogy, we engineered a double-cysteine mutant, GlpPh L66C S300C, on the basis of the outward-facing structure of the wild-type GlpPh (ref. 6). Following cross-linking, we confirmed the outward orientation of the transport domain by crystallography (GlpPh\(^{\text{out}}\); Fig. 1b, Table 1 and Supplementary Fig. 1). Like GlpPh\(^{\text{in}}\), GlpPh\(^{\text{out}}\) shows peaks in the anomalous difference Fourier maps adjacent to the sulfur atoms of the cysteine residues (Supplementary Fig. 1b), which reveal Hg\(^{2+}\) ions in the expected positions and distant from the substrate- and ion-binding sites (Fig. 1b). Structures of GlpPh\(^{\text{out}}\) and GlpPh\(^{\text{in}}\) (ref. 5 and this study) were similar to those obtained in the absence of cross-links\(^6,8\) (with r.m.s. deviations of ~0.9 Å). Isothermal titration calorimetry (ITC) experiments showed that Hg\(^{2+}\) binds the GlpPh L66C S300C and GlpPh K55C A364C protomers with 1:1 stoichiometry and nanomolar affinity in both the substrate-bound and free states (Fig. 1c and Supplementary Table 1). These results confirm that Hg\(^{2+}\) cross-linking traps the mutant transporters in the outward- and inward-facing states, respectively, regardless of whether they are bound to the substrate or not.

Ions drive substrate binding to the isolated states

Using ITC, we showed that aspartate binds with 1:1 stoichiometry and similar affinities to GlpPh\(^{\text{out}}\), GlpPh\(^{\text{in}}\) and the unconstrained wild-type transporter in the presence of 10 mM Na\(^+\) (Fig. 2a). The dissociation constants (K\(_d\)) were 240 ± 50, 290 ± 30 and 480 ± 10 nM (mean ± s.e.m.), respectively. Hence, the transporter has similar intrinsic affinities for its substrate on the opposite sides of the membrane at 25 °C. Similar results have been obtained in other families of ion-coupled transporters\(^2\).

Binding of aspartate to wild-type GlpPh has been shown to be coupled to binding of Na\(^+\) ions\(^6\). We asked whether this coupling is a property intrinsic to the outward- and inward-facing states or whether it requires unrestrained movements of the transport domain. To explore a wide range of binding constants, we optimized a fluorescence-based assay developed in a previous study\(^1,9,20\). A voltagesensitive amphipathic dye, RH421, is incorporated into the proteindetergent particles, and it reports the binding of ligands that alter the local electric field\(^21\). Coupled Na\(^+\) and aspartate binding was measured by detecting the decrease in the emission intensity of the probe (Fig. 2b and Supplementary Fig. 2a). The K\(_d\)s, obtained by

| Table 1 Data collection and refinement statistics |
|-------------------------------------------------|
| **GlpPh\(^{\text{out}}\)**                        |
| Space group                                      |
| P2\(_1\)                                         |
| Cell dimensions                                  |
| a, c (Å)                                        |
| 119.7, 333.7                                    |
| α, β, γ (°)                                     |
| 90.0, 90.0, 120.0                               |
| Resolution (Å)                                  |
| 50.0–4.5 (4.58–4.50)\(^a\)                      |
| R\(_{wp}\)                                        |
| 8.7                                            |
| I/σI                                            |
| 14.5 (2.3)                                     |
| Completeness (%)                                |
| 100 (100)                                     |
| Redundancy                                      |
| 12.6 (12.7)                                    |
| Refinement                                      |
| Resolution (Å)                                  |
| 30–4.5                                         |
| No. reflections                                 |
| 15,187                                         |
| R\(_{work}\) / R\(_{free}\)                      |
| 25.0 / 29.7                                    |
| No. atoms                                       |
| 8,727                                          |
| Protein                                        |
| 8,694                                          |
| Ligand/ion                                     |
| 33                                             |
| B factors                                      |
| 195.9                                          |
| Protein                                        |
| 195.9                                          |
| Ligand/ion                                     |
| 167.7                                          |
| r.m.s. deviations                              |
| Bond lengths (Å)                                |
| 0.004                                         |
| Bond angles (°)                                 |
| 0.8                                            |

\(^{a}\)Values in parentheses are for highest-resolution shell.
Figure 2  Na⁺-coupled binding in the outward- and inward-facing states. Binding data at 25 °C for GltPh<sup>out</sup>, GltPh<sup>in</sup> and wild-type transporter are shown in blue (left), red (center) and black (right), respectively. (a) Aspartate binding isotherms derived from the ITC experiments conducted in the presence of 10 mM Na⁺. Black lines are the fits to the independent-binding-sites model, with the following parameters for the three constructs, respectively: \( K_D = 220, 211 \) and 493 nM; \( \Delta H = -16.4, -17.8 \) and \( -12.3 \) kcal mol<sup>−1</sup>; and apparent number of binding sites, \( n = 0.8, 0.8 \) and 1.0. Insets show the thermal powers for aspartate binding with the corresponding scales. (b) Aspartate titrations derived from fluorescence-based assays in the presence of 1 mM Na⁺ at 25 °C. Solid black lines are fits to the single-site quadratic equation with \( K_D = 125, 232 \) and 457 μM for GltPh<sup>out</sup>, GltPh<sup>in</sup> and wild type, respectively. These lines represent the best fits of the data to equation (1), with the parameters shown next to the graphs. The y axis on the right shows corresponding free energies of binding. \( \Delta G^o \) values are shown in kcal/mol.

Fitting the normalized fluorescence changes, were in excellent agreement with those obtained in ITC experiments (Fig. 2c). The aspartate \( K_D \) of all transporter variants were strongly Na⁺ dependent, and the logarithmic plots of the affinities as a function of Na⁺ activity yielded straight lines with slopes of 2.6, 2.6 and 2.9 for GltPh<sup>out</sup>, GltPh<sup>in</sup> and wild type, respectively. These slopes represent the apparent number of Na⁺ ions coupled to binding of each substrate molecule. Hence, aspartate binding is thermodynamically linked to binding of all three transported Na⁺ ions in wild-type GltPh. Moreover, in the constrained mutants the coupling efficiencies are similar, which shows that transport-domain movements are not necessary. We conclude that on both sides of the membrane essentially all chemical potential of Na⁺ ions is transformed into substrate-binding energy. These results contrast with the coupling mechanisms proposed for other families of secondary active transporters, in which the coupled ions do not change the affinity for the substrate<sup>2</sup> or the substrate can bind in the absence of coupled ions<sup>22</sup>.

Functional studies on the mammalian glutamate transporters have shown that, as with GltPh, three Na⁺ ions are cotransported with each substrate molecule<sup>23–25</sup>. Because of the high degree of conservation of residues involved in the coordination of Na⁺ and substrate between GltPh and the mammalian homologs, it is likely that the efficient coupling between substrate and ions is also conserved. However, glutamate \( K_m \) decreases approximately linearly with the increase of Na⁺ concentration<sup>2,26</sup>, which corresponds to a slope of \( -1 \) on the logarithmic plot. The relative insensitivity of the glutamate \( K_m \) values to Na⁺ concentrations probably reflects the significant contributions from other steps in the cycle following binding, in particular the dissociation of the substrate on the opposite side of the membrane.

Inhibitor captures early events of the binding reaction

In parallel, we examined binding of the inhibitors l-threo-β-benzylaspartate (TBA)<sup>27</sup> and dl-threo-β-benzylxloaspartate (dl-TBOA)<sup>28</sup>. In these experiments, we focused on TBA rather than on the commonly used and structurally similar dl-TBOA because the latter is a mixture of stereoisomers that bind the transporters with different affinities<sup>29</sup> and complicate the analysis. TBA inhibits aspartate transport by GltPh<sup>out</sup> (Supplementary Fig. 2b) and binds the constrained variants and wild-type GltPh, with similar affinities (Fig. 2c). The Na⁺ dependence of TBA-binding affinity was also similar for GltPh<sup>out</sup>, GltPh<sup>in</sup> and wild type and was weaker than that for aspartate, with slopes of 1.9, 2.2 and 1.6, respectively (Fig. 2c).

To confirm that TBA binds the same site as aspartate, we first saturated the transporters with TBA and then titrated them with aspartate by using ITC. As expected for the competitive binding, the free energies and enthalpies of aspartate binding to TBA-loaded transporters agreed with the values calculated for TBA replacement by aspartate when using the individually measured binding parameters (Supplementary Fig. 3 and Supplementary Table 1). We conclude that TBA binds both the outward- and inward-facing states in the substrate-binding pockets, disrupting one of the Na⁺-binding sites. These results are in agreement with previous studies showing that l-TBOA binds the outward-facing GltPh<sup>in</sup> with its backbone in the substrate-binding site and the benzyl group preventing HP2 from occluding the site and forming the Na2<sup>10</sup> site<sup>6</sup>. On the basis of these results, we hypothesize that the TBA-bound states can be viewed as intermediates of the binding reactions, in which two Na⁺ ions and aspartate are already bound but the TBA-bound states can be viewed as intermediates of the binding reactions, in which two Na⁺ ions and aspartate are already bound but not yet occluded. If so, TBA offers a tool to gain insights into the energetics of two processes: the initial binding of the substrate coupled to the third Na⁺ ion. The latter process is represented by the TBA replacement with aspartate.

Na⁺ binding and allosteric coupling

Using the fluorescence-based assay, we also examined Na⁺ binding to GltPh<sup>out</sup> variants in the absence of aspartate and TBA. Na⁺ binds weakly...
Figure 3 Na⁺ binding. (a-c) Na⁺ binding isotherms at 25 °C, derived from the fluorescence-based assays using RH421 dye, for Gltpinh (a), Gltpout (b) and wild type (c) in the absence of other ligands (solid circles) or in the presence of 1 mM aspartate (open squares) or 1 mM TBA (open triangles). Solid black lines are fits to Hill equations. The fitted Kₛ values in mM and Hill coefficients were, respectively: 64.2, 1.6 (no aspartate); 0.2, 1.2 (1 mM aspartate); 5.8, 1.8 (1 mM TBA) (a), 198.0, 0.9; 0.2, 1.5; 4.8, 1.2 (b) and 99.3, 1.6; 0.4, 1.6; 4.3, 1.4 (c). The normalization points for the fits of the data in solid circles were obtained by adding 1 mM aspartate to the cuvette after the Na⁺ titrations to ensure saturation of the Na⁺-binding sites.

To obtain ΔCₚ of the overall reaction, we determined the temperature dependence of the enthalpy (ΔH) of aspartate binding in the presence of Na⁺ concentrations below 50 mM. Under these conditions, there is little ion binding alone, and only the coupled binding of three Na⁺ ions and aspartate is observed. The reaction ΔH decreased linearly with temperature, yielding very large negative ΔCₚ, which was constant within the tested temperature range (Fig. 4). Unexpectedly, we found that ΔCₚ was markedly larger in the inward-facing (−600 cal mol⁻¹ K⁻¹) compared to the outward-facing (−300 cal mol⁻¹ K⁻¹) state. This is in a sharp contrast with the structures, which show nearly identical ion- and substrate-binding sites, and with the similar observed Kₛ at 25 °C. In reactions dominated by protein conformational changes, ΔCₚ scales linearly with the buried polar and apolar surface areas31. If so, the observed ΔCₛ would require burial of ~2,000 and ~4,000 Å² of the protein surface in the outward- and inward-facing states, respectively31. The current models suggest that the release of the substrate from the outward- and inward-facing states is associated with the openings of the local gates: HP2 in the former and a structurally symmetric HP1 in the latter6,7,16–18,34. However, we estimate that HP2 closure buries only ~30 cal mol⁻¹ K⁻¹. In fact, the experimental ΔCₛ are so large that they cannot be explained by protein conformational changes alone. Previously, unexpectedly large ΔCₛ has been attributed to the entrapment of structured waters in the protein core during binding35 to heterogeneous apo state36 and to protein rigidification37 or folding coupled to binding38. Although we cannot distinguish between these possibilities, it is clear that binding involves more complex events in addition to the local conformational changes and that these events are more extensive in the inward-facing state.

Na⁺ binds too weakly to be measured by ITC directly. Instead, we measured ΔH and ΔCₚ of aspartate binding to Gltpinh in the presence of 1 M Na⁺ (Fig. 4). Under these conditions, all sites to which Na⁺ ions bind in the absence of aspartate are occupied (Fig. 3). We observed that aspartate binds Na⁺-saturated transporter with 1:1 stoichiometry and Kₛ in the nanomolar range or lower (Supplementary Fig. 5a). To obtain ΔCₛ of Na⁺ binding, we calculated the differences between ΔCₛ of aspartate binding in the presence and in the absence of 1 M Na⁺ (Supplementary Note and Supplementary Fig. 5b). From this analysis, we found that binding of Na⁺ ions alone is already associated with large negative ΔCₛ and that the differences in ΔCₛ of the coupled binding of three Na⁺ ions and aspartate in the outward- and inward-facing states are almost entirely due to the differences in ion binding.

The ΔH and ΔCₛ values of TBA displacement by aspartate were measured directly (Supplementary Fig. 3b). The ΔCₛ of TBA binding to Na⁺-saturated transporter was then calculated as difference between the corresponding value for aspartate binding in 1 M Na⁺ and TBA displacement (Supplementary Note and Supplementary Fig. 5b).
From these calculations, the $\Delta C_p$ values of TBA binding to Na+-saturated transporter were close to zero in the outward- and inward-facing states (Fig. 5). As expected, similar small $\Delta C_p$ s were calculated for DL-TBOA (Supplementary Fig. 6). These measurements suggest that binding of the substrate backbone, mimicked by the inhibitor, occurs with low $\Delta C_p$, as is typical for a small molecule binding to a preformed site. The $\Delta C_p$ values of aspartate replacement of TBA, which mimics substrate occlusion and binding of the last Na+, were also similar in the outward- and inward-facing states (Fig. 5). Notably, TBA is not likely to bind similarly in the two states: in the outward-facing transport domain, the benzyl group is expected to prop HP2 in an open state, a conformation that would be sterically prohibited when the domain is facing inward. Notably, the $\Delta C_p$ s were again markedly larger than expected for HP2 closure, which suggests that during these steps of the binding reactions, events other than gate closures dominate the processes.

Extrapolations to physiological temperatures

Our experiments were conducted at 25 °C, below the physiological temperatures of ~100 °C at which GltPh normally operates. At 25 °C, GltPh has a very high affinity for aspartate, making it an inefficient enzyme. Because the affinity is strongly dependent on Na+ concentration, the transporter is functional, albeit slow, when reconstituted into proteoliposomes with the internal Na+ concentration near zero (refs. 3,6). In contrast, GltPh should be largely inactive when expressed in mesophilic cells: given the intracellular concentrations of Na+ and aspartate on the order of 10 mM and 1 mM, respectively, only 0.05% of the inward-facing transporters would be substrate free, which would render them highly inefficient.

Many enzymes from thermophilic organisms are either completely inactive or very slow at ambient temperatures but when assayed at their physiological temperatures closely mimic their mesophilic counterparts. To extrapolate the free energies of binding to 100 °C, we used the standard thermodynamic equation

$$\Delta G = \Delta G_{ref} + \Delta C_p(T - T_{ref}) - T \Delta C_p \ln \left( \frac{T}{T_{ref}} \right)$$

where $\Delta G_{ref}$ is the free-energy change measured at $T_{ref} = 25 °C$ (Supplementary Note). Such extrapolation is valid when $\Delta C_p$ does not change significantly with temperature. It is possible that this assumption does not hold and, hence, that the obtained values reflect the general trend rather than precise estimates. Because the heat capacities are markedly different in the outward- and inward-facing state, the aspartate affinities diverge at higher temperatures and are estimated to be 6.3 and 1.400 μM, respectively, at 100 mM Na+ and 100 °C. The affinity for TBA in the outward-facing states is also about 50 times higher than in the inward-facing state (Fig. 6). The decreased affinities at elevated temperatures are mostly due to the progressively less favorable free energy of Na+ binding. In fact, the extrapolations suggest that the transporter does not bind the ions without substrate at all, at least in the absence of the electric field. $K_m$ values of approximately an order of magnitude higher and inhibition constants of two orders of magnitude higher in the inward-facing state have also been measured for glutamate and DL-TBOA.
respectively, in a mammalian homolog\textsuperscript{26}. Lower intracellular affinity for the substrate in both the mammalian and archaeal transporters may reflect an evolutionarily conserved mechanism to optimize the efficiency of substrate transport into the cytoplasm.

**DISCUSSION**

Here, we have shown that binding of aspartate to Glt\textsubscript{Ph} is strictly coupled to binding of all three cotransported Na\textsuperscript{+} ions and that all Na\textsuperscript{+} ions are bound before transmembrane movement of the transport domain takes place. Hence, Na\textsuperscript{+} ions facilitate transport by driving substrate binding and unbinding on the opposite sides of the membrane and not by, for example, binding a translocation intermediate. Furthermore, although aspartate does not bind the transporter with a measurable affinity in the absence of Na\textsuperscript{+}, two Na\textsuperscript{+} ions do bind alone, albeit weakly. Similarly, the mammalian glutamate transporters bind one or two Na\textsuperscript{+} ions weakly in a step that precedes binding of the substrate and the last Na\textsuperscript{+} ion\textsuperscript{42}. Hence, we suggest that binding of the first two Na\textsuperscript{+} ions is a prerequisite for the consequent binding steps. The high cooperativity of aspartate and Na\textsuperscript{+} binding, which is allosteric in nature, suggests that binding of the first two Na\textsuperscript{+} ions is associated with remodeling of the transporter core to form a competent substrate-binding site, which imposes a large energetic penalty on ion binding. Consistently, these early steps in the substrate-binding reactions are associated with very large decreases of the protein heat capacity, which cannot be explained solely by the local conformational changes such as openings or closures of the gates. Instead, large \(\Delta C_P\) values suggest that the conformational changes are more extensive and/or are accompanied by rigidification of the protein or the involvement of water molecules. Consequent binding of the substrate backbone occurs with few additional conformational changes, whereas occlusion of the binding pocket and binding of the third Na\textsuperscript{+} ion is again associated with large \(\Delta C_P\), which is indicative of complex processes. It is notable that the reaction steps associated with Na\textsuperscript{+} binding, and not with complexation of the substrate backbone, produce unexpectedly large heat-capacity changes. To the best of our knowledge, \(\Delta C_P\)\textsubscript{S} associated with ion binding to transport proteins have not been previously reported. However, large \(\Delta C_P\)\textsubscript{S} have been observed in studies of Na\textsuperscript{+} binding to serine proteases and attributed to rigidification of a flexible autolysis loop\textsuperscript{37}. By analogy, we suggest that the large \(\Delta C_P\) associated with Na\textsuperscript{+} binding to Glt\textsubscript{Ph} is due to diminished protein flexibility and that the apo inward-facing state is more dynamic compared to the outward facing state. We conclude that Na\textsuperscript{+} ions do not bind preformed sites but instead trigger conformational changes within the protein and may also have global effects on the dynamic properties of the transporter.

In conclusion, the ability to isolate and study key conformational states of a transporter offers a means to deconstruct the complex transport cycle into simple reactions amenable to quantitative analysis. Using this approach, we show that the outward- and inward-facing states of Glt\textsubscript{Ph} are similar in their ability to couple binding of all three Na\textsuperscript{+} ions to binding of the substrate. However, the molecular events underlying these coupled reactions are distinct and are more complex than expected for a simple ligand-binding process accompanied by local conformational changes. Although further crystallographic studies of the apo states of Glt\textsubscript{Ph} will resolve the structural rearrangements associated with ion-coupled substrate binding, the complementary thermodynamic data highlight the importance of the ensemble properties of these dynamic molecules.

**METHODS**

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

**Access codes.** Coordinates and structural factors have been deposited in the Protein Data Bank, with code 4IZM.

Note: Supplementary information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

N.R. and O.B. designed the experiments. N.R. and S.O. conducted the experiments, and N.R. and O.B. analyzed the data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

DNA manipulations, protein preparation, concentration determination and activity assays. Double-cysteine mutations were introduced within heptahistidine (His8) cysteine-less mutant Glp93 (ref. 5) by using QuikChange Site-directed Mutagenesis Kit (Agilent). Each protein was expressed as C-terminal His8 fusion and purified as described previously43. Briefly, isolated crude membranes from DH10B E. coli overexpressing the protein variants were solubilized in N-dodecyl-β-D-maltopyranoside (DDM) and applied to immobilized metal-affinity resin in buffer containing 10 mM HEPES/KOH, 100 mM NaCl, 0.1 mM aspartate and 0.4 mM DDM. The resin was washed in the same buffer supplemented with 40 mM imidazole, and proteins were eluted in the presence of 250 mM imidazole. The His8 tag was removed by thrombin digestion, and proteins were further purified by size-exclusion chromatography (SEC)5 in appropriate buffers, as indicated below. Protein concentration was determined by absorbance at 280 nm. The extinction coefficient was determined by conducting quantitative amino acid analysis (Keck Biotechnology Resource Laboratory, Yale University). Glp93 L66C S300C, Glp93 K55C A364C and WT Glp93 showed similar results, and the final extinction coefficient used was an average from the three proteins, with a value of 57,400 M⁻¹ cm⁻¹. Notably, this value is approximately two times higher than the value estimated from the sequence (http://web.expasy.org/protparam/). For activity assays, Glp93 was reconstituted into liposomes composed of total polar lipid extract/egg phosphatidylcholine 3:1 (w:w) at 1:50 protein/lipid ratio as described previously46. The proteoliposomes were loaded with 20 mM HEPES/Tris, pH 7.4, and 300 mM choline chloride, and the uptake was measured at 30 °C in buffer containing 20 mM HEPES/Tris, pH 7.4, 200 mM choline chloride, 100 mM NaCl, 0.1 mM [3H]aspartate and increasing concentration of TBA or HgCl2 as described46.

Protein crystallization and structure determination. Prior to crystallization, Glp93 L66C S300C mutant protein was purified by SEC in buffer containing 10 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 0.1 mM aspartate and 7 mM N-decyl-β-D-maltopyranoside. Samples were concentrated to over 5 mg/ml and cross-linked with ten-fold molar excess of HgCl2. Cross-linked protein samples were dialyzed overnight against SEC buffer and crystallized and cryo-protected as described previously46. The diffraction data were collected at 1.075-Å wavelength and cryogenic temperatures at the National Synchrotron Light Source beamline X29. Data were scaled by using HKL2000 (ref. 43) and further processed by using the CCP4 suite45. The initial crystallographic phases were determined in Phaser45, and the electron-density maps were examined in Coot47. Following refinement, 57,839,985 and 2.4% of the backbone dihedral angles were within the core, allowed and disallowed regions of the Ramachandran plot, respectively. All structural figures were prepared with PyMol (www.pymol.org/).

Isothermal titration calorimetry. For ITC experiments, Glp93, K55C A364C and Glp93 L66C S300C were cross-linked following thrombin digest. To avoid chelation of Hg²⁺ ions by imidazole, proteins were exchanged into a buffer containing 10 mM HEPES/KOH, pH 7.4, 50 mM NaCl, 50 mM KCl and 0.4 mM DDM, by using 15-ml filters (Amicon) with a molecular-weight cutoff of 100 kDa. Proteins were concentrated to over 5 mg/ml, incubated with ten-fold molar excess of HgCl2 and purified by SEC using the following buffer: 10 mM HEPES/KOH, pH 7.4, 1 mM NaCl, 99 mM KCl and 0.4 mM DDM. For the binding experiments in 1 M NaCl, the concentration of DDM was increased to 1 mM. WT Glp93 was prepared in the same way except without buffer exchange and HgCl2 addition. Quantitative amino acid analysis of the nonhydrolyzed protein samples showed no detectable aspartate or other free amino acids in the samples, indicating that this protocol generates protein completely unbound from its substrate. Protein samples diluted to 15–40 µM in buffer supplemented with appropriate concentrations of NaCl were loaded into the reaction cell of a small-volume NANO ITC (TA Instruments). Appropriate titrant was loaded into the syringe, and aliquots of 1.5 µl were injected every 3–5 min at constant temperature. For the competition experiments, the syringe was loaded with 200–300 µM aspartate, 600 µM TBA and 30 mM NaCl and the cell with 40 µM protein, 600 µM TBA and 30 mM NaCl. Binding isotherms were analyzed with NanoAnalyze (TA Instruments) using the independent-binding site-model.

Fluorescence-based binding assays. For fluorescence-based binding assays, Glp93 samples were prepared as above and diluted to final concentration of 0.5–1 µM in the same buffer as for the ITC experiments but using 0.2 mM instead of 0.4 mM DDM. The binding assays were performed at 25 °C in the presence of 200 mM of RH421 dye (Invitrogen). The fluorescence was excited and the emission detected at 532 nm and 628 nm, respectively, using a QuantaMaster 40 (Photon International Technology). The protein solutions with dye were allowed to equilibrate under illumination for ~15 min until the fluorescence signal was stable. Aliquots of NaCl, aspartate or TBA were added to the protein, and the reaction mixtures were incubated until no additional fluorescence signal change could be detected. Binding isotherms were analyzed with SigmaPlot 12 (Systat Software). For each ligand addition, the fluorescence signal acquired during the last 10 s of equilibration was averaged. The maximum signal change was determined by addition of 1 mM aspartate to the samples at the end of each titration. The fractional fluorescence change was calculated as the difference between measured and initial fluorescence normalized by the maximum fluorescence change. Aspartate or TBA titrations were fitted to the quadratic binding equation to take into account substrate depletion at low Kd whereas Na⁺titrations were fitted to the Hill equation. When using the latter, care was taken that the measured Kd was at least 100-fold higher than the protein concentration used. Na⁺ activities were calculated as activity = γ×[Na⁺], where γ is the activity coefficient calculated with the Debye-Hückel equation, log10 γ = A/[1 + B·|I|], where A and B are empirical coefficients with values 0.51 and 1.5, respectively, and I is the ionic strength of the solution.