Conformational heterogeneity of the aspartate transporter GltPh

Inga Hänelt1, Dorith Wunnicke2, Enrica Bordignon3, Heinz-Jürgen Steinhoff2 & Dirk Jan Slotboom1,4

GltPh is a Pyrococcus horikoshii homotrimeric Na⁺-coupled aspartate transporter that belongs to the glutamate transporter family. Each protomer consists of a trimerization domain involved in subunit interaction and a transporting domain with the substrate-binding site. Here, we have studied the conformational changes underlying transport by GltPh using EPR spectroscopy. The trimerization domains form a rigid scaffold, whereas the transporting domains sample multiple conformations, consistent with large-scale movements during the transport cycle. Binding of substrates changed the occupancies of the different conformational states, but the domains remained heterogeneous. The membrane environment favored conformations different from those observed in detergent micelles, but the transporting domain remained structurally heterogeneous in both environments. We conclude that the transporting domains sample multiple conformational states with substantial occupancy regardless of the presence of substrate and coupling ions, consistent with equilibrium constants close to unity between the observed transporter conformations.

RESULTS
Design of EPR measurements
To monitor the structural changes that occur upon substrate binding by EPR spectroscopy, we used site-directed spin labeling. We created single and double cysteine mutants of GltPh that we modified with the reagent 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methylmethanethiosulfonate (MTSL). Figure 1 gives an overview of the residues used for site-directed spin labeling. Transport assays using the purified proteins in proteoliposomes showed that all spin-labeled mutants were active in aspartate transport (Fig. 1e).

We used a pulsed EPR method (double electron-electron resonance (DEER)) with a working temperature of 50 K to measure distances of 1.5–8 nm between the spin-labeled side chains, and we used continuous wave (CW) EPR experiments with a working temperature...
We analyzed the effects of three-spin articles and spin labeling are color-coded: purple, Thr166; orange, Val176; blue, Ser300; yellow, Ser331; red, Ala364; cyan, Lys55.

of 160 K to qualitatively extract information on interspin distances <1.8 nm (ref. 14). We analyzed GltPh both solubilized in detergent micelles and reconstituted in proteoliposomes and compared the results from each experiment type.

The trimerization domain is a stable scaffold

Introduction of a spin label at a single position in GltPh allows for the measurement of the distance between the protomers of the homotrimeric protein. We constructed two trimerization-domain mutants by replacing Thr166 (located in the cytoplasmic end of transmembrane helix 4) and Val176 (located in the cytoplasmic end of helix 5) with an MTSL-modified cysteine (R1). Simulations on the available crystal structures indicated that these spin labels are >1.8 nm apart and therefore suitable for DEER measurements\(^ {15}\). We did not observe spectral broadening in the CW EPR measurements, which further supports the notion that the spin labels were >1.8 nm apart (Supplementary Fig. 1). We recorded spectra of the apoprotein and of the protein in the presence of saturating concentrations of Na\(^+\) alone or Na\(^+\) and aspartate. In agreement with previous biochemical and structural data\(^ {12,13}\), the trimerization domain did not show any large-scale conformational changes upon addition of coupling ions or substrate (Fig. 2). We observed two sharp peaks (centered around 2.8 nm and 3.8 nm for T166R1 and 2.6 nm and 3.4 nm for V176R1) in the interspin distance distributions for proteins in the presence and absence of substrates. The measured distances were in agreement with the interprotomer distances calculated on the basis of the crystal structures (Table 1 and Fig. 2).

Because GltPh is a trimer, each mutation resulted in the presence of three cysteines per protein complex, which could result in a considerable population with three spin labels per trimer, depending on the efficiency of spin labeling (Supplementary Table 1). In such cases, the experimental distance distributions obtained by DEER may contain artifacts\(^ {16}\). We analyzed the effects of three-spin artifacts for the spin-labeled mutants used here (Supplementary Fig. 2) and found that they contributed only marginally to the observed experimental distributions.

The transporting domain is conformationally heterogeneous

The mutants S300R1, S331R1 and A364R1 are labeled in the transporting domain at the N-terminal end of transmembrane segment 7, the C-terminal end of transmembrane segment 7 and the C-terminal half of HP2, respectively. The available crystal structures suggest that the intermolecular interspin distances in these mutants differ between the outward- and inward-oriented states. The distance between Ser300 residues from different protomers is shorter in the outward- than in

**Figure 1** Residues selected for EPR measurements. (a–d) Ribbon diagrams of GltPh trimers as viewed from the cytoplasm (a,b) and GltPh protomers as viewed from the membrane plane (c,d). TM, transmembrane segment. Black lines (c,d) indicate the position of the plasma membrane. Transporting domains are shown in the outward-facing orientation (2NWL) (a,c) and the inward-facing orientation (3KBC) (b,d). The residues used for cysteine mutagenesis and spin labeling are color-coded: purple, Thr166; orange, Val176; blue, Ser300; yellow, Ser331; red, Ala364; cyan, Lys55.

**Figure 2** Interprotomer distances in the trimerization and the transporting domains in detergent solution. (a,b) DEER data of solubilized GltPh variants recorded at X band (9.4 GHz; for mutants T166R1, V176R1 and S300R1) or Q band (34 GHz; for mutants S331R1 and A364R1). Data are shown for the apoprotein (black lines) and for the protein in the presence of Na\(^+\) (NaCl; red lines) or Na\(^+\) and aspartate (NaCl, Asp; blue lines). Normalized background-corrected dipolar evolution data \( F(t) \) are shown in a; tick marks are separated by 0.1. Distance distributions obtained by Tikhonov regularization and by a rotamer library analysis\(^ {19}\) of the inward-facing (i, dotted lines) and outward-facing (o, dashed lines) crystal structures are shown in b.
Table 1 Theoretical and experimental distances in the EPR measurements

<table>
<thead>
<tr>
<th>Residue</th>
<th>Experimental mean interspin distance (nm)</th>
<th>Co-Co (nm)</th>
<th>Rotamer library approach (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt;2 (s)</td>
<td>&gt;2 (45%)</td>
</tr>
<tr>
<td>T166R1</td>
<td>3.86 (s)</td>
<td>3.81 (s)</td>
<td></td>
</tr>
<tr>
<td>V176R1</td>
<td>3.41 (s)</td>
<td>3.46 (s)</td>
<td></td>
</tr>
<tr>
<td>S300R1</td>
<td>4.21 (s)</td>
<td>4.54 (s)</td>
<td></td>
</tr>
<tr>
<td>S331R1</td>
<td>4.68 (s)</td>
<td>5.8 (s)</td>
<td></td>
</tr>
<tr>
<td>A364R1</td>
<td>3.79 (s)</td>
<td>3.08 (s)</td>
<td></td>
</tr>
<tr>
<td>K55R1 A364R1</td>
<td>&gt;2 (75%), &gt;2 (45%), &gt;2 (38%), &gt;2 (20%)</td>
<td>1.3 (25%)</td>
<td></td>
</tr>
<tr>
<td>K55R1 A364R1</td>
<td>&lt;1.2*, &lt;1.2*</td>
<td>1.3 (80%)</td>
<td></td>
</tr>
</tbody>
</table>

Experimentally determined mean interspin distances, derived from DEER analysis (for single mutants) or from DIPFIT (for K55R1 A364R1), for the detergent-solubilized (s) and liposome-reconstituted (r) apoprotein (–) or protein in the presence of Na+ alone or Na+ and aspartate. Cases in which CW spectra were recorded and no dipolar broadening was observed were assigned a distance of >2 nm. For K55R1 A364R1, the percentage of the fractions with and without dipolar broadening is given in brackets. Distance values estimated from the spectral second moment (denoted with asterisk) indicate the presence of interspin distances <1.2 nm. Distance distribution width was fixed to 0.3 nm for the fittings of the CW EPR spectra. Co-Co, distances as shown by the crystal structures 10,12; rotamer library approach, corresponding major distances resulting from the rotamer library approach as implemented in MmM (o, outward-oriented conformation; i, inward-oriented conformation).

The membrane environment affects the protein conformations

All measurements described above were performed using detergent-solubilized proteins. To explore the effects of the environment on the conformational transitions of GltPh, we reconstituted spin-labeled mutants in liposomes. We selected GltPh variants carrying mutations V176R1 (located in the trimerization domain of GltPh), S331R1 or A364R1 (located in the transporting domain) and the K55R1 A364R1 double mutant to study distance distributions in proteoliposomes (Figs. 3 and 4). V176R1 showed the same distance distribution in the membrane as in detergent solution (Table 1 and Supplementary Fig. 4), regardless of substrate binding. In contrast, for mutants S331R1 and A364R1, the interspin distances were different in proteoliposomes compared to detergent solution. Both mutants showed heterogeneous conformational behavior. For both mutants and in detergent solution, the inward-facing state in the presence of Na+ alone and Na+ and aspartate. Both in the presence and in the absence of substrate, the experimentally determined distances were broader than those calculated from the crystal structures, which could indicate that the trimerization and transporting domains are more loosely associated in solution than in the crystals or that additional conformations not represented by the available crystal structures are present.

Intra-protomer distance measurements

The double cysteine mutant K55C A364C has been used to solve the crystal structure of inward-oriented GltPh through cross-linking of the two residues using Hg2+ (ref. 12). To further study the relative occupancy of the different conformational states of GltPh, we spin labeled K55C A364C and used CW EPR lineshape analysis to obtain distance information. This technique is less sensitive than DEER for determining multiple distance distributions, but in the K55R1 A364R1 variant, the distance between the spin labels is expected to be <1.8 nm only in the inward-oriented conformation (Supplementary Fig. 3c); therefore, it was possible to selectively measure the occupancy of this conformational state by CW EPR. In contrast, DEER analysis of the doubly spin-labeled mutant is complicated by the overlap of intra- and interprotomer distances (Supplementary Fig. 3).

The spectra of the doubly spin-labeled mutant were dipolar broadened compared to the sum of singly labeled mutants, in the absence of substrate and in the presence of Na+ or Na+ together with aspartate (Fig. 3), indicating that a fraction of spin labels were <2 nm apart in all cases. The spectra showed an increased broadening in the presence of Na+ as compared to the apo state, which was even more pronounced in the presence of both Na+ and aspartate. The increase in dipolar broadening indicates a decrease in the distance, which indicates that a higher fraction of the protein is in the inward-facing conformation.
distance distributions in detergent and in the membrane, but the extreme
distances were suppressed in the membrane, and intermediate
distances appeared. For mutant A364R1, the mean distances between
the spin labels decreased with addition of coupling ions or substrate
(4.2 nm in the apo state, 3.6 nm in presence of Na\(^+\) and 3.5 nm with
Na\(^+\) and aspartate present) (Fig. 4 and Table 1). The changes were
qualitatively similar to those observed in micelles; however, the change
in mean distance (\(\Delta d\)) was only –0.7 nm in proteoliposomes compared
\(\Delta d\) to –0.9 nm in the solubilized protein (Table 1). This observation
indicates that the main conformational changes were smaller in liposomes
and that some of the extreme conformations observed in the crystal
structure are sampled less frequently in the membrane environment.
We observed a similar effect for mutant S331R1, in which the mean
distance increased from 5.3 nm to 5.7 nm with substrate and coupling ion
addition, resulting in a \(\Delta d\) of 0.4 nm, smaller than that found in
micelles (\(\Delta d = 1.3 \text{ nm}\)) (Table 1). The notion that the extreme confor-
mations are sampled less frequently in the membrane environment is
also supported by the intra-protomer measurements of spin distances
in mutant K55R1 A364R1. Notably, the presence of strong spin-spin
(dipolar and Heisenberg) interactions in the apoprotein indicates dis-

tances in the 1-nm range (Fig. 3). Binding of Na\(^+\) alone or Na\(^+\) and
aspartate reduced the spectral broadening, indicating an increase in
average interspin distances. Thus, in mutant K55R1 A364R1 in pro-
teoliposomes, substrate addition induced opposite distance changes
compared to K55R1 A364R1 in micelles, highlighting the role of the
environment in the stabilization of particular conformations.

**DISCUSSION**

The EPR measurements show that the trimerization domains form
the stable core of trimeric GltpH, both in detergent solution and in a
membrane environment. The rigidity of the trimerization domain is
consistent with crystallographic and functional data\(^\text{12,13}\). In contrast,
the transporting domains are not captured in a single conformation;
rather, they sample multiple conformations, in both the presence and
absence of substrate (aspartate) and coupling ions (Na\(^+\)). In detergent
solution, GltpH samples the inward-facing (3KBC) and outward-facing
(2NWL) conformations, consistent with the conformations caught in
the crystal structures. In contrast, different, intermediate conforma-
tions were preferred in the membrane, and these could be similar to
those found in an asymmetric crystal structure\(^\text{9}\) in which the pro-
tomers of the trimer were in different conformations. Occupancy of
the conformational states was affected by binding of coupling ions
and substrate, as well as by the detergent or lipid environment, but
multiple conformational states were sampled in all conditions tested.
These observations suggest that the equilibrium constants between
the observed transporter conformations are close to unity and, con-
sequently, that different states are populated to similar extents. This
observation makes sense for the empty carrier and for GltpH bound to
both Na\(^+\) and aspartate\(^\text{17}\), as these forms need to be able to isomerize
between outward- and inward-facing states for substrate binding and
release. However, the Na\(^+\)-bound protein would not be expected to
alternately expose the binding site, as this would lead to uncoupled
Na\(^+\) leaks. Nonetheless, multiple conformational states were sam-
ped in the Na\(^+\)-loaded carrier as well. Our interpretation of the EPR
results is that the transporting domain is constantly shuttling across
the membrane, a prerequisite for alternately exposing the binding
site. We suggest that local rearrangements like those shown for HP2
(refs. 12,18) trigger the alternating accessibility of the binding sites by
coordinately opening and closing the inward- and outward-facing lids
or gates, and that the gating is dependent on the presence of substrate
and coupling ions. Cross-linking studies on human EAAT1 support
the observation that large conformational changes take place in both
the presence and the absence of substrates\(^\text{19}\).

Notably, the binding of coupling ions and substrate resulted in
apparently opposite effects in different spin-labeled mutants in the
transporting domain. In mutants S300R1 and A364R1, binding of
both Na\(^+\) alone and Na\(^+\) and aspartate caused an increase in occu-
pancy of the inward-oriented state as compared to the apoprotein,
whereas mutant S331R1 showed a change in the opposite direction.
The occupancy of the states is apparently sensitive to insertion of a
cysteine, modification by spin labeling or, possibly, to the freezing
that is required to extract distance information by EPR. Again, these
observations are consistent with an equilibrium constant close to
unity between the observed transporter conformations, a condition in
which small perturbations may affect the population of the states.

The available crystal structures of GltpH have yielded unprece-
dented insight into the transport mechanism by providing snapshots
of extreme and intermediate conformations. We have compared the
conformations of GltpH in detergent solution and membranes and
have concluded that the large conformational changes suggested by
the crystal structures can also be observed in these environments, but
that the membrane and the micelle favor different conformations.
Moreover, we find that subtle modifications in the protein can have
pronounced effects on the equilibrium of the outward- and inward-
faceconformations.

**METHODS**

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

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

We thank R.H. Duurkens for performing uptake experiments, C. Rickert, D. Klose and J. Klare for help with the EPR measurements and B. Poolman for constructive criticism. This work was supported by a research fellowship and by a research grant from the Deutsche Forschungsgemeinschaft (HA 6322/1-1 to I.H. and STE 640/10, SFB944 to D.W. and H.-J.S.), the Netherlands Organisation for Scientific Research (NWO Vidi and Vici grant to D.J.S.) and the European Union (EDIT program and European Research Council starting grant to D.J.S.).

AUTHOR CONTRIBUTIONS

I.H. and D.J.S. designed the experiments. I.H., D.W. and E.B. conducted the experiments. All authors contributed to writing the manuscript and analyzing the data.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nsmb.2471.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

ONLINE METHODS

Mutagenesis, expression, purification and labeling of GltPh. Cysteine mutants were introduced in recombinant GltPh, possessing a C-terminal eight-histidine tag using site-directed mutagenesis. DNA sequencing confirmed the presence of only the desired mutations. GltPh, was overproduced in *Escherichia coli* MC1061 and purified as previously described, with slight modifications. Buffer A containing 50 mM Tris-HCl (pH 8.0), 300 mM KCl and 0.04% (wt/vol) N-dodecyl-β-D-maltopyranoside (DDM) (Anatrace) was used throughout the purification process and subsequent washes with degassed buffers only. Protein bound to Ni²⁺-Sepharose was labeled overnight at 4 °C with 1 mM (1-oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl)methylmethanethiosulfonate spin label (MTSL) (Toronto Research Chemicals, Inc.). Subsequently, size-exclusion chromatography was performed to remove free spin label. Before labeling, protein concentrations were determined using the calculated extinction coefficients.

Protein reconstitution and transport of [¹⁴C]aspartate into proteoliposomes. Reconstitution of GltPh mutants into proteoliposomes and transport of [¹⁴C]aspartate were performed as previously described, with the following modifications: spin-labeled cysteine mutants were reconstituted into a mixture of synthetic lipids (Avanti Polar Lipids, Inc.) of a 3:1:1 weight ratio of 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC); 1,2-dioleoyl-sn-glycerol-3-phospho-(1′-rac-glycerol) (DOPG). The transport of aspartate was initiated by diluting 2 µl of proteoliposomes (125 µg/µl lipid concentration) loaded with 50 mM KPi buffer (pH 7) into 200 µl 50 mM sodium phosphate buffer (pH 7) containing 0.69 µM [¹⁴C]aspartate and 0.5 µM valinomycin.

EPR measurements. Spin-labeled mutants were concentrated via Vivaspin columns (cutoff 100 kDa, Sartorius) to 100–200 µM and washed two times with buffer A in D₂O supplemented when indicated with 1 M NaCl or 1 M NaCl and 100 µM aspartate. For spin-labeled mutants reconstituted into liposomes, the proteoliposomes were suspended to a concentration of ~100 µM in 50 mM potassium phosphate buffer containing 0.5 µM valinomycin in D₂O (pH 7). 1 M NaCl, or 1 M NaCl and 100 µM aspartate, were present when indicated. Liposomes were frozen in liquid nitrogen immediately after resuspension. X-band CW EPR spectra at room temperature were recorded using a homemade EPR spectrometer equipped with a dielectric resonator (Bruker) (1 mW microwave power; 0.15 mT B-field modulation amplitude). Sample volumes of 10 µl were placed in EPR glass capillaries. X-band CW EPR spectra at 160 K were carried out using a homemade EPR spectrometer equipped with a Super High Sensitivity Probehead (Bruker) (0.2 mW microwave power; 0.25 mT B-field modulation amplitude), a continuous flow helium cryostat (Oxford Instruments) and a temperature controller (Oxford Instruments). An RMN 2 B-field meter (Drusch) allowed measurement of the magnetic field. Sample volumes of 30–40 µl were loaded into EPR quartz capillaries and frozen in liquid nitrogen before insertion into the resonator.

Pulsed EPR experiments were performed at X band at 50 K on an Elexys 580 spectrometer (Bruker) equipped with a continuous-flow helium cryostat (Oxford Instruments) in combination with a temperature controller (Oxford Instruments). The four-pulse DEER sequence was applied. All parameters were kept unmodified with respect to previously described values, with observer pulses of 16–32 ns and a pump pulse of 12 ns. Sample volumes of 30–40 µl were loaded into EPR quartz tubes (3 mm outer diameter) and shock frozen in liquid nitrogen. The same 3-mm tubes were used to measure Q-band DEER at 50 K in a homemade spectrometer equipped with a 150 W TWT (traveling-wave tube) and an oversized resonator. All pulses were set to 12 ns, and the frequency separation was set to 100 MHz (ref. 23).

Fitting of experimental CW EPR data. Simulated dipolar broadened EPR spectra were fitted to experimental low temperature CW EPR spectra considering a Gaussian distribution of interspin distances using DipFit. Best-fit parameters for interspin distance distributions were determined. The g tensor values, the A_xx and A_yy values of the hyperfine tensor and the Lorentzian and Gaussian linewidth parameters were fixed to values found for the reference spectra of mutant A364R in detergent-solubilized and lipidosome-reconstituted forms, respectively. EPR spectra were convoluted with a field-independent lineshape function composed of a superposition of 50% Lorentzian and 50% Gaussian shapes of widths 0.37 mT and 0.32 mT, respectively.

DEER analysis. The DEER traces were background corrected using a homogeneous three-dimensional spin distribution. Interspin distance distributions were derived by fitting the background-corrected dipolar evolution function using Tikhonov regularization as implemented in DEERAnalysis2011 (ref. 25). Validation of distance distributions was carried out with the included validation tool. For the mutant A364R in detergent micelles, which showed an invariant peak at about 6 nm in all conditions, changes to the background dimensionality during validation resulted in a pronounced variability of the peak intensity. We thus consider the 6-nm peak to be affected by background artifacts, and we did not consider it in the calculation of the mean distances in Table 1. The lower spin concentration of the reconstituted samples allowed for the extraction of a reliable background function in all cases, and the validation confirmed the distance distribution presented.

Rotamer library approach. Distance distributions were simulated using a rotamer library approach (RLA) as implemented in MMM. The rotamer library consists of 210 precalculated rotamers representing an ensemble of possible spin-label side chain (R1) conformations at 175 K. The orientation of R1 introduced at the chosen residue with regard to the protein structure permits calculation of the energy for the R1-protein interaction in consideration of the Lennard-Jones potential. Multiplication of the probability for each rotamer (determined by Boltzmann weighting and normalization by the partition function) with the probability that R1 will exhibit this conformation leads to the rotamer probability distribution for a specific residue. Interspin distance distributions are calculated as the histogram of all pairwise interspin distances weighted by the product of their respective probabilities. The three-spin effects on the DEER F(t) traces were also calculated with MMM, and the simulated F(t) traces were subsequently analyzed with DeerAnalysis2011 to obtain the artifacts on the distance distribution for 100% labeling efficiency.