On the mechanism of prokaryotic glutamate transporter homologues
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Chapter 4

Na⁺: aspartate coupling stoichiometry in the glutamate transporter homologue Glt<sub>Ph</sub>

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Abstract

The Na⁺-aspartate symporter Glt<sub>Ph</sub> from Pyrococcus horikoshii is the only member of the glutamate transporter family for which crystal structures have been determined. The cation:aspartate coupling stoichiometry is unknown, thus hampering the elucidation of the ion coupling mechanism. Here we measured transport of $^{22}$Na⁺ and $[^{14}$C]aspartate in proteoliposomes containing purified Glt<sub>Ph</sub> and demonstrate that three Na⁺ ions are symported with aspartate.
Introduction

Glutamate transporters belong to a ubiquitous family of cation-coupled membrane transporters (95). In humans, five subtypes of glutamate transporters have been found (excitatory amino acid transporters, EAAT1-5) that pump the neurotransmitter glutamate from the extracellular fluid into cells (16). In prokaryotes, the substrates of the transporters serve as carbon, nitrogen or energy source (102). Glutamate transporters use the free energy stored in electrochemical membrane gradients of cations (sodium ions, potassium ions and/or protons) to pump glutamate uphill against its concentration gradient. Electrophysiological experiments with human EAAT3 and rat EAAT2 have demonstrated that three sodium ions and one proton are cotransported with each glutamate molecule. To reset the empty carrier in the outward facing conformation, one potassium ion is subsequently translocated in the opposite direction (68, 125). In 2004, the first crystal structure of a homologous archaeal aspartate transporter (GltPh) was published (121). GltPh couples aspartate transport to Na⁺ symport (9) and does not use K⁺ or protons for co- or countertransport (90). The protein is a good model for studying the transport mechanism of EAATs, because the residues that have been implicated in glutamate and cation binding are well-conserved (121), and the fold is consistent with a large body of mutagenesis studies on mammalian EAATs (58, 92). The available experimental data on the cation coupling stoichiometry indicate that at least two sodium ions are co-transported with aspartate, but the exact Na⁺:aspartate coupling stoichiometry is not known (9, 90, 91). Determination of the stoichiometry for GltPh is of major importance, since the protein functions as a model for the entire family, and the number is crucial to elucidate the mechanism of cation coupling in the glutamate transporter family.

Methods

Chemicals

[¹⁴C]aspartate (1.85 Mbq/mL, 8.06 Gbq/mmol) was purchased from GE Healthcare (USA). ²²Na⁺ (993.83 MBq/mL, 63.717 Gbq/mg) was obtained from Perkin-Elmer (Boston MA, USA). All chemicals were of analytical grade.

DNA manipulations

The gene encoding GltPh was amplified from plasmid pET25-cGFP(G4) (kind gift from E. Gouaux) using KOD polymerase (Merck KGaA, Darmstadt DE) according to the manufacturer’s protocol. Primers were designed to allow LIC (ligation independent cloning), as has been described by Geertsma et al (35). The constructed expression plasmid was a pBAD24 derivative, harbouring the gene encoding GltPh with an N-terminal His₁₀ tag and a TEV cleavage site between the tag and the protein.

Purification and reconstitution of GltPh

The expression vector was transformed to E. coli MC1061 cells and the recombinant strain was cultivated in LB medium supplemented with 100 µg/mL ampicillin. At an optical density of 0.8 at 660 nm, expression was induced
with 0.01% L-arabinose. Two hours after induction cells were harvested (10,000 rcf, 10’, 4°C, Beckman JLA 8.1000 rotor) and resuspended in 20 mM Tris-HCl (pH 8), DNAse (10 µg/mL), PMSF (phenylmethanesulfonyl fluoride, 200 µM) and MgSO4 (1 mM) were added, and cells were passed once through a cell disrupter cooled at 4°C and operated at 20 kPsi (Constant Systems Ltd. Daventry UK). Unbroken cells and cell debris were pelleted (5000 rcf, 25’, 4°C, Harrier 18/80 swing out centrifuge) and the supernatant was subjected to ultracentrifugation (150,000 rcf, 90’, 4°C, Beckman 50.2ti rotor). Membrane pellets were resuspended in 20 mM Tris-HCl (pH 8), and stored at -80°C. The protein concentration in the membranes was determined using Bradford reagent, with BSA as a standard. For protein purification, membrane vesicles were solubilized in buffer A (50 mM Tris-HCl (pH 8.0), 300 mM KCl), containing 15 mM imidazole pH 8.0 and 1% n-dodecyl-β-D-maltopyranoside (DDM), at a final protein concentration of 5 mg/mL. After incubation on ice for 45’, with occasional gentle shaking, the solution was centrifuged (267,000 rcf, 15’, 4°C, Beckman TLA 100.4 rotor). Supernatants were incubated on a rotating platform for 60’ at 4°C with nickel-sepharose slurry (0.5 ml bed volume, Fast-flow, GE Healthcare), pre-equilibrated with buffer A containing 15 mM imidazole pH 8.0. The mixture was loaded on a Biorad Poly-Prep column and unbound protein was allowed to flow through. Columns were washed with 20 column volumes of buffer A, supplemented with 60 mM imidazole and 0.15 % n-dodecyl-β-D-maltopyranoside (DM). Protein was eluted from the column in three fractions of 300, 500 and 500 µl respectively, using buffer A supplemented with 500 mM imidazole pH 8.0 and 0.15% DM. The second elution fraction from the affinity chromatography contained most of the purified protein and was loaded directly on a Superdex-200 gelfiltration column, using 20 mM potassium phosphate pH 7.0, 150 mM KCl, 0.15% DM as eluens. The entire purification and reconstitution protocol was performed in buffers devoid of Na+ and aspartate, so that empty, substrate free protein was obtained (87). The concentration of purified Glt,α was determined by measuring the absorbance at 280 nm (extinction coefficient 283 10 M⁻¹ cm⁻¹)

For reconstitution in liposomes, a 3:1 (w:w) mixture of E. coli polar lipid extract and egg PC (Avanti polar lipids, Alabaster USA) was prepared. Liposomes were homogenized by extruding 9 times through a 400 nm pore size polycarbonate filter. Liposomes were subsequently diluted in 50 mM potassium phosphate pH 7.0, to a final concentration of 4 mg/mL. Liposomes were destabilized by step-wise addition of Triton X-100 (10% w/v). The absorption (scattering) at 540 nm was monitored, and titration was stopped when the absorbance had decreased to 2/3 of the maximum absorbance (34). Purified protein was added in a 1:250 protein:lipid ratio (w/w). The reconstitution mixture was incubated at room temperature for 30’, under gentle movement. Biobeads (34) were added (25 mg/mL), followed by another 30’ incubation at room temperature. After addition of more biobeads (15 mg/mL), the temperature was lowered to 4°C, and the mixture was incubated for 60’ under gentle movement. Biobeads were added again (19 mg/mL), followed by overnight incubation at 4°C. Subsequently a final amount of biobeads was added (29 mg/mL), followed by 120’ incubation at 4°C. Biobeads were removed, and proteoliposomes were subjected to ultracentrifugation (267,000 rcf, 4°C, 20’, Beckman TLA 100.4 rotor). Pellets were resuspended in 50 mM potassium phosphate pH 7.0, to a final lipid concentration of 20 mg/mL. Proteoliposomes were subjected to three cycles of freeze thawing using liquid nitrogen, and subsequently stored in liquid nitrogen.

**Transport assays using proteoliposomes**

Proteoliposomes were thawed, extruded as described above and centrifuged (267,000 rcf, 4°C, 20’, Beckman TLA 100.4 rotor). For transport assays, 2 µL of proteoliposomes resuspended in the internal buffer (125 µg/µL lipid concentration) were diluted into 200 µL 50 mM potassium phosphate containing 5 mM NaCl and 0.5 µM valinomycin (from a 3 mM stock in ethanol). The use of equal concentrations of potassium ions on either side of the membrane in combination with the K⁺ ionophore valinomycin ensured that the membrane potential was clamped at 0 mV. The external buffer was supplemented with (i) 0.71 µM 22Na⁺ and 4.2 µM aspartate (to measure 22Na⁺ uptake), or (ii) 2.8 µM aspartate supplemented with 1.4 µM [14C]aspartate (to measure [14C]aspartate uptake). External buffers were pre-equilibrated at 30°C in plastic tubes (CBN, Drachten the Netherlands), and after subsequent addition of proteoliposomes the mixture was briefly vortexed. The reactions were stopped by...
addition of 2 mL ice-cold 150 mM KCl, followed by suction filtration over Optitran BA-S 85 filters (Whatman Maidstone UK), followed by 5 washing steps with 2 mL 150 mM KCl each. To determine the background counts for $^{22}$Na we added 0.71 µM $^{22}$Na to the external buffer, from which aspartate had been omitted, and the same procedure was followed as in the presence of aspartate. The background counts for [14C]aspartate were determined either by omitting Na$^+$ from the external buffer, or in the presence of Na$^+$ by immediately stopping the uptake reaction at timepoint zero. Both methods yielded the same background counts within the experimental error.

Radioactivity trapped on the filters was measured by addition of 2 mL of emulsifier plus scintillation liquid, and subsequent counting in a Perkin Elmer 1600CA scintillation counter. To determine the amount of aspartate taken up by the proteoliposomes, we measured the total amount of radioactivity in the uptake assay (corresponding to 840 pmol aspartate (200 µL of 4.2 µM aspartate)) and calculated the percentage of aspartate taken up. Alternatively, we determined the efficiency of our scintillation counter in the presence of filters (89%), and used this number together with the specific activity to calculate how much aspartate had been internalized in the proteoliposomes. Both methods yielded the same number within the experimental error. To determine the amount of transported sodium, we measured the total amount of radioactivity in the uptake assay (corresponding to 1 µmol Na$^+$ (200 µL of 5 mM Na$^+$)) and calculated the percentage of Na$^+$ taken up. Alternatively, we determined the efficiency of our scintillation counter for $^{22}$Na$^+$ (100%), and used this number together with the specific activity (corrected for decay as indicated by the supplier) to calculate how much Na$^+$ had been internalized in the proteoliposomes. Again, both methods yielded the same number within the experimental error.

**Results**

The electrophysiological methods that were used to determine the cation:glutamate coupling stoichiometry of eukaryotic glutamate transporters cannot be applied to bacterial cells. Therefore, we used another generic method to determine the Na$^+$:aspartate stoichiometry of Glt$_{ph}$, measurement of the uptake of both [14C]aspartate and $^{22}$Na$^+$ in proteoliposomes containing purified and reconstituted protein. While transport of radiolabeled aspartate by Glt$_{ph}$ can be readily measured by rapid filtration assays (9, 90, 91) (figure 1A), it proved to be difficult to obtain accurate transport numbers for Na$^+$ transport because of excessive scatter in the data. These problems have been reported in the literature previously (45) and also have been experienced by others (J. Mindell, personal communication, anonymous peer reviewers of grant proposals).

We made four adaptations to the rapid filtration transport assay (9, 31, 91) to improve the reproducibility of the $^{22}$Na$^+$ uptake assays. First, the level of nonspecific binding of $^{22}$Na$^+$ to the filters was reduced by using filters with low background binding (Optitran nitrocellulose BA-S 85). Second, we replaced borosilicate glass tubes with disposable plastic tubes. Borosilicate glass is known to interact with sodium ions (52), and the scatter in our $^{22}$Na$^+$ assays was dramatically reduced when plastic tubes were used. Third, transport by Glt$_{ph}$ is electrogenic (91), and transport rates and accumulation levels are increased by a negative membrane potential. However, the application of a negative membrane potential (K$^+$ diffusion potential created by an outward gradient of K$^+$ in the presence of the ionophore valinomycin) resulted in a substantial Na$^+$ leak in our transport experiments,
even when liposomes were used that did not contain any protein. When we clamped the membrane potential to 0 mV, the leaks disappeared. Fourth, the use of phosphate buffers yielded better signal-to-noise ratios than MES/HEPES buffers. Besides these technical considerations, the sensitivity of the transport assay also complicated the $^{22}\text{Na}^+$ uptake measurements. As will be shown below by a calculation, it was necessary (1) to maximize the amounts of $\text{Na}^+$ and aspartate transported into the liposome lumen and (2) to use $^{22}\text{Na}^+$ and $[^{14}\text{C}]$aspartate of sufficient specific activity to accurately measure the internalized isotopes by scintillation counting. The upper limit of substrate accumulation is determined thermodynamically by the concentration gradients of $\text{Na}^+$ and aspartate across the membrane and by the electrical membrane potential. We created large concentration gradients by omitting aspartate and $\text{Na}^+$ from the luminal buffer and using high external concentrations (5 mM Na$^+$ and 4.2 μM aspartate (see below for a justification of these concentrations)). As discussed above, a negative membrane potential could not be used; instead, we clamped the membrane potential at 0 mV. The amount of liposomes used in the assay also affected the maximal amount of substrate that could be internalized, because they determine the luminal volume. In practice, the rapid filtration assay is not compatible with excessive amounts of liposomes because the filters become blocked. The optimal amount of liposomes used in the transport assay was 250 μg of lipids (internal volume of ~0.23 μL) (34).

Although the kinetics of transport do not affect the accumulation levels that can be achieved thermodynamically, high uptake rates were favourable because (1) they reduce the assay time and (2) thermodynamic equilibrium may not be achieved in slow transport assays. The rate of uptake is affected by the protein concentration (protein:lipid ratio in the liposomes) and the external substrate concentrations. A protein:lipid ratio of 1:250 (w/w) was found to be optimal in the transport assays. Higher protein concentrations gave lower specific transport activities and compromised the seal of liposomes (producing leaks). The aspartate and Na$^+$ concentrations were set well above the $K_m$ values for transport: 5 mM external Na$^+$ (2–3-fold higher than the $K_m$) and 4.2 μM aspartate (>10-fold greater than $K_m$) (90). High external substrate concentrations also increased the concentration gradients across the membrane, which favorably affected the accumulation levels.
Figure 1: Transport of Na⁺ and aspartate in proteoliposomes containing purified and reconstituted Gltᵦᵣ. (A) Typical transport curve for [¹⁴C]aspartate. The luminal buffer was 50 mM potassium phosphate (pH 7.0); the external buffer consisted of 50 mM potassium phosphate (pH 7.0), 5 mM NaCl, 0.5 μM valinomycin, and 4.2 μM [¹⁴C]aspartate. (B) Uptake of [¹⁴C]aspartate and ²²Na⁺ was assessed separately at two time points (3 and 6 min) using two independent batches of purified and reconstituted Gltᵦᵣ, and the Na⁺:aspartate ratio was determined. Uptake of each isotope at each time point was assessed seven or eight times; error bars indicate standard deviations. (C and D) Raw data (counts per minute) for the assays at the 6 and 3 min time points, respectively. Empty and filled symbols indicate background and transport measurements, respectively. Gray drop lines show the means. To calculate the ratios shown in panel B from the raw data, the mean background was subtracted from the mean transport data (using common error propagation of the standard deviations). The counts were converted to picomoles via the total count measurements (n = 5) or using the specific activity data. The calculated amount of Na⁺ transported was divided by the amount of aspartate transported to obtain the ratios. All standard deviations were propagated according to standard rules. The counts per minute and picomole values for the experiments shown in panels A, C, and D cannot be compared directly, because slightly different amounts of total radioactivity were used, as well as independently prepared batches of proteoliposomes with small differences in the specific transport activities.
Under these conditions, sensitivity was not a problem for the uptake of $[^{14}\text{C}]$aspartate (figure 1A). For example, in a typical transport assay, $\sim85$ pmol of aspartate accumulated in the proteoliposomes after 3 min (figure 1A). With a specific activity of $\sim2.7$ GBq/mmoll for $[^{14}\text{C}]$aspartate, this amounted to $\sim13770 \pm 280$ dpm ($n = 8$) (corresponding to $\sim12250$ cpm using a scintillation counter with a counting efficiency of 89%), which was well above the background ($\sim470 \pm 30$ dpm ($n = 4$)). However, for $^{22}\text{Na}^+$, the situation was very different. At the external $\text{Na}^+$ concentration of 5 mM, only a low specific activity of $^{22}\text{Na}^+$ could be used ($\sim0.2$ GBq/mmol). Higher levels of radioactivity were not affordable and would produce excessive radiation. Even at the relatively low specific $^{22}\text{Na}^+$ activity that was used, the total activity of $^{22}\text{Na}^+$ ($\sim200000$ Bq) in the assay was already $\sim100$-fold higher than the $[^{14}\text{C}]$aspartate activity (2200 Bq). Using these conditions and assuming a $\text{Na}^+$:aspartate coupling stoichiometry of 2:1 or 3:1, only a few thousand disintegrations per minute of $^{22}\text{Na}^+$ would be counted at the 3 min time point when $\sim85$ pmol of aspartate was internalized (figure 1A). With a nonspecific background of 5450 $\pm$ 830 dpm ($n = 16$ (figure 1C,D)), this number would be close to the lower limit of what can be measured accurately. The calculation shows that only for longer time points in the uptake curve (figure 1A) sufficient amounts of substrate could be internalized to accurately measure $^{22}\text{Na}^+$ uptake. We thus decided to measure the amounts of sodium ions and aspartate transported at the 3 and 6 min time points. We found $\text{Na}^+$:aspartate ratios of 3.38 $\pm$0.31 ($n = 8$) and 2.91 $\pm$0.21 ($n = 7$) at these time points, respectively, using two independent batches of purified and reconstituted Glt$_{ph}$. When all data were combined, an average ratio of 3.15 $\pm$ 0.35 for the $\text{Na}^+$:aspartate stoichiometry was found (figure 1B).

**Conclusions**

We conclude that three sodium ions are symported with each molecule of aspartate by Glt$_{ph}$. The $\text{Na}^+$ coupling stoichiometry is the same as in the mammalian glutamate transporters and thus makes Glt$_{ph}$ a suitable model protein for studying $\text{Na}^+$ coupling in EAATs. Our finding shows that, next to the two binding sites for sodium ions found by thallium ion replacement in the Glt$_{ph}$ crystal structure (9), a third site has to be present as well. Holley and Kavanaugh have postulated the presence of an additional binding site on the basis of electrostatic modeling of the human EAAT3 protein modeled on the Glt$_{ph}$ structure (48). It is possible that this additional binding site is present in Glt$_{ph}$ as well, since the two other $\text{Na}^+$ binding sites found by Holley and Kavanaugh overlapped with the thallium ion binding sites (48).
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