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Na^+:Aspartate Coupling Stoichiometry in the Glutamate Transporter Homologue Glt_{Ph}[†]

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ABSTRACT: The Na^+ aspartate symporter Glt_{Ph} 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 measure transport of $^{22}Na^+$ and [^{14}C]aspartate in proteoliposomes containing purified Glt_{Ph} and demonstrate that three Na^+ ions are symported with aspartate.

Glutamate transporters belong to a ubiquitous family of cation-coupled membrane transporters (*1*). 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 (*2*). In prokaryotes, the substrates of the transporters serve as a carbon, nitrogen, or energy source (*3*).

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 (*4*, *5*). In 2004, the first crystal structure of a homologous archaeal aspartate transporter (Glt_{Ph}) was published (*6*). Glt_{Ph} couples aspartate transport to Na^+ symport (*7*) and does not use K^+ or protons for co- or countertransport (*8*). 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 (*6*), and the fold is consistent with a large body of mutagenesis studies on mammalian EAATs (*9*, *10*). The available experimental data on the cation coupling stoichiometry indicate that at least two sodium ions are cotransported with aspartate, but the exact Na^+:aspartate coupling stoichiometry is not known (*7*, *8*, *11*). Determination of the stoichiometry for Glt_{Ph} 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.

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 [^{14}C]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 (*7*, *8*, *11*) (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 (*12*) 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 (*7*, *11*, *13*) to improve the reproducibility of the $^{22}Na^+$ uptake assays (see the Supporting Information for a detailed description of the methods). 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 (*14*), and the scatter in our $^{22}Na^+$ assays was dramatically reduced when plastic tubes were used. Third, transport by Glt_{Ph} is electrogenic (*11*), 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 $^{22}Na^+$ uptake measurements. As will be shown below by a calculation, it was necessary (1) to maximize the amounts of Na^+ and aspartate transported into the liposome lumen and (2) to use $^{22}Na^+$ and [^{14}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 Na^+ and aspartate across the membrane and by the electrical membrane potential. We created large concentration gradients by omitting aspartate and Na^+ from the luminal buffer and using high external concentrations [5 mM Na^+ and 4.2 μM asparate (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

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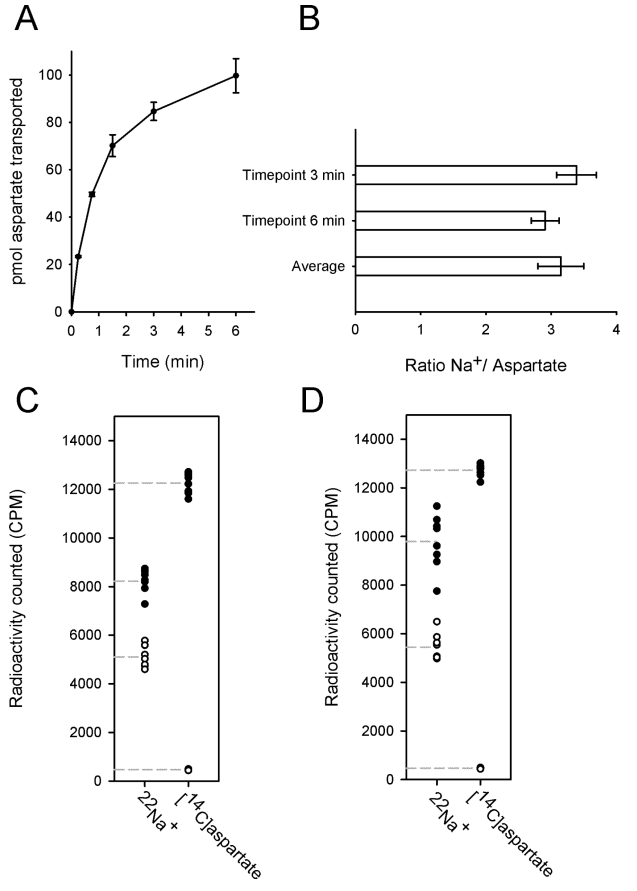

FIGURE 1: Transport of Na^+ and aspartate in proteoliposomes containing purified and reconstituted Glt_{Ph}. (A) Typical transport curve for [^{14}C]aspartate. The luminal buffer was 50 mM potassium phosphate (pH 7.0); the external buffer consisted of 50 mM potassium phosphate (pH 7.0), 5 mM NaCl, 0.5 μM valinomycin, and 4.2 μM [^{14}C]aspartate. (B) Uptake of [^{14}C]aspartate and $^{22}Na^+$ was assessed separately at two time points (3 and 6 min) using two independent batches of purified and reconstituted Glt_{Ph}, 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.

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 (*15*)].

Although the kinetics of transport do not affect the accumulation levels that can be achieved thermodynamically, high uptake rates were favorable 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) (*8*). High external substrate concentrations also increased the concentration gradients across the membrane, which favorably affected the accumulation levels.

Under these conditions, sensitivity was not a problem for the uptake of [^{14}C]aspartate (Figure 1A). For example, in a typical transport assay, ~85 pmol of aspartate accumulated in the proteoliposomes after 3 min (Figure 1A). With a specific activity of ~2.7 GBq/mmol for [^{14}C]aspartate, this amounted to ~13770 ± 280 dpm (n = 8) (corresponding to ~12250 cpm using a scintillation counter with a counting efficiency of 89%), which was well above the background [~470 ± 30 dpm (n = 4)]. However, for $^{22}Na^+$, the situation was very different. At the external Na^+ concentration of 5 mM, only a low specific activity of $^{22}Na^+$ could be used (~0.2 GBq/mmol). Higher levels of radioactivity were not affordable and would produce excessive radiation. Even at the relatively low specific $^{22}Na^+$ activity that was used, the total activity of $^{22}Na^+$ (~200000 Bq) in the assay was already ~100-fold higher than the [^{14}C]asparate activity (2200 Bq). Using these conditions and assuming a Na^+:aspartate coupling stoichiometry of 2:1 or 3:1, only a few thousand disintegrations per minute of $^{22}Na^+$ would be counted at the 3 min time point when ~85 pmol of aspartate was internalized (Figure 1A). With a nonspecific background of 5450 ± 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 $^{22}Na^+$ uptake. We thus decided to measure the amounts of sodium ions and aspartate transported at the 3 and 6 min time points. We found Na^+:aspartate ratios of 3.38 [±0.31 (n = 8)] and 2.91 [±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 ± 0.35 for the Na^+:aspartate stoichiometry was found (Figure 1B).

We conclude that three sodium ions are symported with each molecule of aspartate by Glt_{Ph}. The Na^+ coupling stoichiometry is the same as in the mammalian glutamate transporters and thus makes Glt_{Ph} a suitable model protein for studying 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 (*7*), 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 (*16*). It is possible that this additional binding site is present in Glt_{Ph} as well, since the two other Na^+ binding sites found by Holley and Kavanaugh overlapped with the thallium ion binding sites (*16*).

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SUPPORTING INFORMATION AVAILABLE

Methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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