Excitatory amino acid transporters (EAATs) are crucial in maintaining extracellular levels of glutamate, the most abundant excitatory neurotransmitter, below toxic levels. The recent three-dimensional crystal structure of GltPh, an archaeal homolog of the EAATs, provides elegant structural details of this family of proteins, yet we know little about the mechanism of the bacterial transporter. Conflicting reports in the literature have described GltPh as an aspartate transporter driven by Na\(^+\) or a glutamate transporter driven by either Na\(^+\) or H\(^+\). Here we use purified protein reconstituted into liposomes to thoroughly characterize the ion and substrate dependence of the GltPh transport. We confirm that GltPh is a Na\(^+\)-dependent transporter that is highly selective for aspartate over other amino acids, and we show that transport is coupled to at least two Na\(^+\) ions. In contrast to the EAATs, transport via GltPh is independent of H\(^+\) and K\(^+\). We propose a kinetic model of transport in which at least two Na\(^+\) ions are coupled to the cotransport of each aspartate molecule by GltPh, and where an ion- and substrate-free transporter reorients to complete the transport cycle.

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system; it activates a wide range of ionotropic and metabotropic receptors to generate synaptic responses. The extracellular glutamate concentration is controlled by a family of specific transport proteins, the EAATs\(^3\) (1), which take up the neurotransmitter into glia and neurons. Dysfunction of EAAT proteins leads to elevations in extracellular glutamate concentrations which, if prolonged, can result in excitotoxicity and neuronal cell death. Defective function and regulation of EAATs have been implicated in multiple human diseases, including amyotrophic lateral sclerosis and Alzheimer disease (1).

The glutamate transporter family includes five human EAAT subtypes (EAAT1–5), two neutral amino acid transporters, and several prokaryotic homologs (2). Glutamate transport via EAATs is coupled to the cotransport of three Na\(^+\) ions and one H\(^+\) and the countertransport of one K\(^+\) ion (3). In addition to this coupled transport, Na\(^+\)-dependent glutamate binding to the EAATs activates a thermodynamically uncoupled anion conductance (4–8). The detailed mechanism of ion coupling and glutamate transport by the EAATs remains unclear; complementary methods are required to understand the physical/chemical features of the transport mechanism.

A recent crystal structure of a homolog of the glutamate transporter family from Pyrococcus horikoshii (GltPh), revealed its complex transmembrane topology (9). GltPh shares about 36% amino acid identity with the EAATs. Many of the residues that have been implicated in glutamate and ion binding/translocation (10–12) and chloride permeation (13) in the EAATs are highly conserved throughout the family, suggesting that the basic architecture of the bacterial and mammalian proteins is very similar. The identification of binding sites for substrate and two Na\(^+\) ions in GltPh (14) also agrees with biochemical experiments demonstrating the functional importance of the highly conserved C-terminal domain in bacterial (15, 16) and mammalian (17–23) transporters.

To fully understand the mechanistic implications of the GltPh structure, we must first understand the basic functional properties of the transporter. Only those mechanistic features that are conserved between prokaryotic and eukaryotic homologs can be understood in detail by analyzing GltPh as a model structure. This sort of functional analysis of GltPh remains at a preliminary stage. An emerging consensus is that GltPh functions as a Na\(^+\)-dependent aspartate transporter (14, 24), although a single report suggests that the protein is H\(^+\)-dependent glutamate transporter (25). We recently demonstrated that, like the EAATs, GltPh carries an uncoupled Cl\(^-\) conductance, and its transport is electrogenic (24); however, its dependence of transport on other ionic species has not yet been examined. Boudker et al. (14) reported an analysis of Na\(^+\) dependence of aspartate binding to detergent-solubilized GltPh protein, but the dependence of transport on substrate and Na\(^+\) concentration has not yet been analyzed. This is an important distinction, as the conditions most favorable for substrate binding to the transporter in isolation may be quite different from those required for optimal transport.

Here we sought to characterize in detail the ionic and substrate requirements for transport in GltPh, measuring transport kinetics of reconstituted protein in a wide range of conditions to determine which features of the GltPh transport mechanism are shared with the EAATs. We also performed a comprehen-
sive analysis of substrate specificity. We demonstrate that Glt$_{ph}$ is a Na$^+$-dependent, highly aspartate-selective transporter. We also show that, in contrast to the EAATs, H$_2$O and K$^+$ are not coupled to aspartate transport by Glt$_{ph}$; based on these results we suggest a simple model for the Glt$_{ph}$ transport cycle.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Reconstitution**—Single cysteine residues were introduced using the QuickChange II site-directed mutagenesis kit (Stratagene) into a Glt$_{ph}$ mutant in which the single native cysteine (Cys-321) had been mutated to a serine (C321S). Mutations were confirmed by sequencing of both DNA strands (NINDS Sequencing Facility, National Institutes of Health). Glt$_{ph}$ protein was purified essentially as described elsewhere (24) with the following modifications. Membranes were isolated, solubilized with n-dodecyl β-D-maltopranoside (Anatrace), and protein was purified using nickel-nitrilotriacetic acid resin (Qiagen). The histidine tag was subsequently removed by digestion with thrombin (10 units/mg protein), and the protein was further purified on a size exclusion column where the detergent was exchanged to n-decyl β-D-maltopyranoside (Anatrace).

Pure protein was reconstituted into liposomes as described previously (26). Briefly, *Escherichia coli* polar lipids and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids), at a ratio of 3:1, were mixed, dried under nitrogen, and resuspended in 100 mM K$_p$, pH 7.4, or the appropriate inside solution as indicated. Liposomes were formed by extrusion through 400 nm membranes (Avanti Polar Lipids) and were treated with Triton X-100 (Anatrace) at a 0.5:1 (w/w) detergent to lipid ratio prior to the addition of protein at 0.25–3 μg of protein/mg of lipid. The protein/lipid was left at room temperature for 30 min before detergent was removed using SM2 Bio-Beads (Bio-Rad) that had been extensively prewashed with methanol and water. The protein/lipid mixture was incubated with gentle agitation, with three consecutive batches of Bio-Beads (80 mg/ml) as follows: the first batch for 2 h at room temperature, the second batch for 2 h at 4 °C, and the third batch overnight at 4 °C. Bio-Beads were removed by filtration. Proteoliposomes were concentrated, resuspended at 100 mg of lipid/ml (0.3–5.0 mg protein/ml), and either used immediately or flash-frozen in a dry ice/EtOH bath and stored at −80 °C.

**Transport Assay**—Generally, proteoliposomes were loaded by freeze/thaw and extrusion with 100 mM KCl, 20 mM HEPES, pH 7.5, and the uptake reaction was initiated by diluting the proteoliposomes (100 mg of lipid/ml) 133-fold into reaction buffer pre-warmed to 30 °C, or as indicated for the temperature experiment. The reaction buffer contained the following: 100 mM NaCl, 20 mM HEPES, pH 7.5, 1 μM valinomycin (except for experiments shown in Fig. 5, see below) and 100 nM, or 1 μM where indicated, L-$[^3]$H]aspartate or L-$[^3]$H]glutamate (GE Healthcare). ChCl was used to maintain ionic and osmotic strength where appropriate. The intraliposomal cation dependence of Glt$_{ph}$ was examined by replacing the KCl in the inside buffer with 100 mM of the chloride salts Rb$^+$, Cs$^+$, Ch$^+$, tetraakis$, CH$, and 200 mM sorbitol; uptake was measured in the absence of valinomycin. Background levels of L-$[^3]$H]aspartate uptake in protein-free liposomes or in Glt$_{ph}$-liposomes with equimolar K$^+$ or Na$^+$ on either side of the membrane were indistinguishable from one another (data not shown). At each time point, a 200-μl aliquot was removed and diluted 10-fold into ice-cold quench buffer (100 mM LiCl, 20 mM HEPES, pH 7.5), followed by immediate filtration over nitrocellulose filters (0.22 μm pore size, Millipore). The filters were washed once with 2 ml of ice-cold quench buffer and assayed for radioactivity using a Trilux beta counter (PerkinElmer Life Sciences). All initial rates were calculated from the linear portion of the curve (first 30 s), and all data represent the means ± S.E. of at least three experiments. Uptake data are fit to single exponentials for presentation.

The efflux assay was performed by first loading the liposomes with L-$[^3]$H]aspartate; Glt$_{ph}$-liposomes containing 100 mM NaCl, 20 mM HEPES, pH 7.4, and 1 μM unlabeled L-aspartate were diluted 20-fold into loading buffer (100 mM NaCl, 20 mM HEPES, pH 7.4, 400 nM L-$[^3]$H]aspartate) and incubated for 3 h at 30 °C. Because K$^+$ is replaced with other ions in these experiments, no valinomycin was added; transport-generated voltages were dissipated by the intrinsic Cl$^-$ conductance of Glt$_{ph}$ (24). Efflux experiments were initiated by a 14-fold dilution of Glt$_{ph}$-liposomes into efflux buffer at 22 °C (100 mM NaCl, KCl, ChCl, or 200 mM sorbitol, 20 mM HEPES, pH 7.5). For counterflow experiments, Glt$_{ph}$-liposomes containing 100 mM NaCl, 20 mM HEPES, pH 7.4, and either 1 μM L-aspartate or 1 mM L-glutamate were diluted 133-fold into buffer at 30 °C (100 mM NaCl, 20 mM HEPES, pH 7.4, 200 mM L-$[^3]$H]aspartate). For both efflux and counterflow, a 200-μl aliquot was taken at each time point and diluted 10-fold into ice-cold quench buffer, followed by immediate filtration over nitrocellulose filters as described above. For the efflux experiment, background counts (taken with protein-free liposomes) were subtracted from the raw data, which were then normalized to the 5-s time point to account for variation in the efficiency of L-$[^3]$H]aspartate loading. This background subtraction had no qualitative effect on the results. The correction was never more than 10% of the initial value.

**Fluorescein Maleimide Labeling of Cysteine Mutants**—Proteoliposomes containing Glt$_{ph}$ were incubated with fluorescein-5-maleimide (Molecular Probes) at a protein/label ratio of 1:1000 for 8 min in a buffer containing 20 mM HEPES/Tris, pH 7.4, 100 mM NaCl, 5 mM L-aspartate. Triton X-100 was added at a 2.5 (w/w) detergent to lipid ratio to solubilize the vesicles either before or after the labeling reaction. The reaction was quenched by addition of L-cysteine at a 1:10 ratio of label to cysteine. Protein was removed from free label by addition of nickel-nitrilotriacetic acid beads and 40 mM imidazole to solubilized proteoliposomes; the beads were washed twice, protein eluted with 250 mM imidazole, and then run on an SDS-polyacrylamide gel. Fluorescent labeling was visualized on a UV transilluminator and then the protein stained with Coomassie Blue (Invitrogen Simply SafeStain Blue) and visualized by white light illumination.

**RESULTS**

**Orientation of Reconstituted Glt$_{ph}$ in Liposomes**—The aim of this study was to characterize the transport mechanism of Glt$_{ph}$
Functional Analysis of Glt<sub>Ph</sub>

FIGURE 1. Orientation of reconstituted Glt<sub>Ph</sub>. A, fluorescein-5-maleimide labeling and Coomassie Blue staining of Glt<sub>Ph</sub> mutants, Cys-less (C321S), and with a single cysteine on the extracellular (G34C) and intracellular (S72C) faces of the protein, in liposomes before (L) and after Triton X-100 solubilization (S) of liposomes. Protein had been reconstituted into vesicles swollen with Triton X-100 at a ratio 0.5 (w/w) detergent to lipid. Top panel represents gel imaged on a UV light box; bottom panel represents the same gel, subsequently stained with Coomassie Blue and imaged with visible transillumination. B, swelling of vesicles measured by light scattering of vesicles (absorbance at 540 nm) as a function of Triton X-100 to lipid ratio. C, gels of labeling experiments as described in A but reconstituted at Triton/lipid ratios of 0.25 (w/w) or in the absence of Triton X-100 (0 w/w).

in detail to understand the ion dependence of transport and to determine which mechanistic features are conserved between this bacterial homolog and the human glutamate transporters. To this end, we performed transport assays on purified Glt<sub>Ph</sub> protein reconstituted into liposomes. To effectively interpret kinetic studies of transport in reconstituted protein, one must first assess the orientation of the protein in the liposome membrane. If the protein is uniformly oriented then detailed kinetic measurements can provide insight into specific steps in the transport cycle. Alternatively, if the protein reconstitutes into vesicles with mixed orientations, then under a given set of ionic gradients a subset of individual proteins, oriented one way around, will transit the transport cycle in the opposite direction compared with the proteins oriented the other way around. In this case, ion dependence of transport can be examined, but detailed kinetic analysis is more difficult.

We analyzed the orientation of reconstituted Glt<sub>Ph</sub> by taking advantage of our ability to specifically label introduced cysteines on the protein with a maleimide-coupled fluorescent probe. Glt<sub>Ph</sub> has a single native cysteine, which can be removed without noticeably changing the functional properties of the protein (data not shown). Into this “Cys-less” Glt<sub>Ph</sub>, we introduced cysteines individually at positions predicted by the crystal structure to be exposed either on the intracellular (S72C) or extracellular (G34C) face of the protein. If the protein is uniformly oriented in the membrane, then when in liposomes either all or none of a given cysteine will be accessible and thereby labeled by the aqueous reagent. Alternatively, if there is a mix of orientations then a fraction of the protein will be labeled. In this case, adding detergent to permeabilize the liposomes before addition of the fluorescent probe will result in increased labeling as the otherwise protected cysteines inside the liposomes become accessible to the aqueous reagent. The outcome of this labeling procedure for a uniformly oriented protein will vary depending on the orientation; if all of the introduced cysteines are outside, all will label in unpermeabilized vesicles; alternatively, if the cysteines are all on the inside, none will label in unpermeabilized vesicles, but all will label after permeabilization. Running the protein on a polyacrylamide gel

and exciting the fluorescent probe provides a convenient semi-quantitative assay for the extent of labeling; the same gel can be stained with Coomassie Blue after imaging to allow for corrections based upon the amount of protein loaded. In detergent-solubilized protein, both internal and external sites react well with the fluorescent probe (data not shown).

Our experiments reveal a reactivity pattern that demonstrates a mixed set of orientations for Glt<sub>Ph</sub> in liposomes. Cys-less, C321S, protein is minimally labeled by fluorescein maleimide under these conditions, demonstrating that the labeling reaction is specific for cysteine (Fig. 1A, top image, right lanes). Both for the external site (Fig. 1A, top image, middle lanes) and the internal site (Fig. 1A, top image, left lanes), we observe significant labeling by the probe with Glt<sub>Ph</sub> in intact liposomes, as well as a substantial increase in labeling upon permeabilization and labeling. Staining the same gel with Coomassie Blue (Fig. 1A, bottom image) verifies consistent loading of protein. These results clearly establish that the protein is present in liposomes in both possible orientations.

Our method for protein reconstitution is derived from that of Rigaud, in which pre-formed lipid vesicles are incubated with enough detergent to incorporate into the vesicle membrane without completely solubilizing it (27). The concentration of detergent added in this protocol is guided by the light-scattering properties of the sample (Fig. 1B). At low concentrations of detergent the scattering increases as the liposomes swell due to detergent molecules intercalating into the bilayer; higher concentrations of detergent start to dissolve the bilayer and reduce the amount of scattering. We initially obtained these results with liposomes that had been substantially solubilized with Triton X-100 before adding protein (0.5 (w/w) detergent/lipid; see asterisk on light-scattering curve in Fig. 1B). We reasoned that it might be possible to obtain a more uniformly oriented population of transporters by reconstituting at lower concentrations of added detergent. However, we obtain similar results with protein added to liposomes solubilized with substantially lower concentrations of Triton (Fig. 1C, top image), including with no added detergent (besides that in the protein solution). These results suggest that the detergent carried in micelles with the Glt<sub>Ph</sub> protein is sufficient to promote its incorporation into a lipid bilayer membrane and that this incorporation is equally efficacious in either orientation. In this context, detailed kinetic analysis of transport is not feasible; however, examinations of the ionic dependence and specificity of transport can be performed to better understand the mechanism of transport by Glt<sub>Ph</sub>.

Glt<sub>Ph</sub> Is a Na<sup>+</sup>-dependent Aspartate Transporter—As reported previously, in the presence of an inwardly directed Na<sup>+</sup> gradient, but no H<sup>+</sup> gradient, we observe robust uptake of
L-[3H]aspartate (125-fold over background), whereas levels of L-[3H]glutamate uptake are indistinguishable from background (Fig. 2A). These results agree with that of Boudker et al. (14), who did not observe any appreciable glutamate uptake over background in the same conditions. A dose response of the initial rate of L-[3H]aspartate uptake reveals an apparent $K_m$ for transport of $120 \pm 30$ nM, Hill coefficient of $1.0 \pm 0.1$, and $V_{max}$ of $3.7 \pm 0.3$ nmol/mg protein/min (Fig. 2B). The half-saturation of aspartate transport rate ($K_m$) is ~100-fold lower than the $K_d$ value reported for aspartate binding to GltPh, consistent with a slow off-rate of binding, which is likely to be comparable with the rate of transport (see “Discussion”). A Hill coefficient of $~1$ agrees well with binding results (14) and suggests independence of substrate translocation pathways in each protomer of the trimeric complex. Studies have reported similar Hill coefficient values for the EAATs (28, 29), supporting the hypothesis that the fundamental transport mechanism is conserved.

To further investigate the substrate specificity of GltPh, we performed competition experiments, measuring uptake of L-[3H]aspartate in the presence of 100-fold excesses of a series of unlabeled amino acids. Of the 20 naturally occurring amino acids, only aspartate itself competes successfully with L-[3H]aspartate uptake, with its L-isomer a slightly better competitor than its D-isomer (Fig. 2C). We also measured aspartate uptake in the presence of several known transportable and nontransportable inhibitors of the EAATs. At concentrations 100-fold over L-[3H]aspartate, only two of the tested compounds, TBOA and cysteate, significantly inhibited aspartate transport via GltPh (Fig. 2C). TBOA, an analog of aspartate, is a selective competitive inhibitor of the EAATs (30), which also inhibits aspartate transport by GltPh (14). At a concentration of $10 \mu M$, we also observed ~70% inhibition of L-[3H]aspartate transport by TBOA. Cysteate, an EAAT substrate (2), inhibits L-[3H]aspartate transport by ~40%, an observation consistent with the binding measurements of Boudker et al. (14) obtained using the similar compound, L-cysteinesulfonic acid. None of the other compounds we tested significantly inhibited aspartate transport.

A recent paper by Raunser and colleagues (25) reports that GltPh is a glutamate transporter that can be driven by gradients of either Na$^+$ or H$^+$. In contrast to their results, we do not observe detectable uptake of L-[3H]glutamate in the presence of a Na$^+$ gradient (at symmetrical pH 7.5, Fig. 2A). Furthermore, a 100-fold excess of unlabeled glutamate does not compete with L-[3H]aspartate transport via GltPh (Fig. 2C). Thus, in our hands, a Na$^+$ gradient alone cannot support glutamate transport via GltPh.

$Na^+$ and H$^+$ Dependence of GltPh Transport—For the mammalian glutamate transporters, three Na$^+$ ions are coupled to

![FIGURE 2. Substrate specificity of GltPh. A, L-[3H]aspartate transport (•) by GltPh in the presence of an inwardly directed Na$^+$ gradient at symmetrical pH 7.5. L-[3H]Glutamate uptake (○) in these conditions overlays that measured for aspartate in the absence of a Na$^+$ gradient (▼). B, initial rate of uptake as a function of L-[3H]aspartate. Data fit to the Hill equation yield a $K_m$ of $120 \pm 30$ nM, a $V_{max}$ of $3.7 \pm 0.3$ nmol/mg protein/min, and a Hill coefficient of $1.0 \pm 0.1$. Same data are plotted as a Haynes plot in the inset. C, initial rates of uptake in the presence of 100 nM [3H]aspartate (normalized to no addition, n.a.) and 10 $\mu M$ unlabeled competitor as indicated on the y axis. All amino acids were L-isomers except *D* (o-aspartate), 1, TBOA; 2, cysteate; 3, serine O-sulfate; 4, kainate; 5, dihydrokainate; 6, (±)-threo-3-methylglutamate; 7, (25R)-4-methylglutamate; 8, homocysteate.](image-url)
Functional Analysis of GltPh

[Diagram showing normalized H-L-aspartate uptake rate as a function of Na⁺ concentration.]

On face value it is difficult to harmonize our data with the results of Raunser et al. (25). We considered the possibility that, in contrast to its Na⁺-dependent aspartate transport, GltPh could utilize a H⁺ gradient to drive glutamate (instead of aspartate) into liposomes. On the scale of Fig. 4A, we do not observe any noticeable glutamate uptake by GltPh in the presence of an inwardly directed H⁺ gradient (pH 7.5 in, 5 out) either alone or in addition to a Na⁺ gradient (Fig. 4A). However, close examination of the glutamate uptake data from Fig. 4A (Fig. 4C, note expanded scale) reveals that a minute amount of glutamate uptake does indeed occur in our system; its level is ∼3-fold over background, in contrast to the level of aspartate uptake, which is ∼125-fold over background. This level of uptake is of similar magnitude to that measured by Raunser et al. (25). Thus, the small amount of glutamate transport we observed in the presence of 100 mM L-[3H]glutamate agrees with both previous studies on GltPh, one reporting a binding affinity of glutamate ∼100 μM (14) and the other reporting a glutamate transport Kₘ of 194 μM (25). In addition, we observe uptake of radiolabeled aspartate under counterflow conditions with saturating concentrations of either L-aspartate (1 μM) or L-glutamate (1 mM) inside the liposomes but not with 1 μM glutamate (data not shown).

We conclude that GltPh functions primarily as an aspartate transporter that has a strict requirement for Na⁺. In our hands a H⁺ gradient, alone or in combination with a Na⁺ gradient, does not drive aspartate transport. Although GltPh can bind and transport glutamate, and glutamate can support counterflow, this activity is insignificant compared with its aspartate transport activity.

K⁺ Dependence of Transport—Glutamate transport via the EAATs is coupled to the countertransport of one K⁺ (3, 31, 32), which is required for the reorientation of the transporter to the extracellular side of the membrane. We investigated the K⁺ dependence of aspartate transport by GltPh, by replacing the K⁺ inside the liposome with the alternate monovalent cation Ch⁻ and measuring L-[3H]aspartate uptake. If, as in the EAATs, K⁺ countertransport is required, this substitution would be expected to significantly inhibit transport. Replacing K⁺ in the inside buffer with Ch⁻ does not affect the rate of L-[3H]aspartate transport (Fig. 5A). This is surprising, given the EAAT requirement of K⁺ to reorient, so we investigated the ability of a series of cations of various sizes and the uncharged sugar sorbitol to support L-[3H]aspartate uptake. When internal K⁺ is replaced with Rb⁺, Cs⁺, Ch⁻, or the large cation tetrais, L-[3H]aspartate uptake is not different from uptake in the presence of internal K⁺ (Fig. 5B). Note that because we are removing K⁺ for these experiments, they are performed without valinomycin; the intrinsic Cl⁻ conductance is sufficient to dissipate the transporter-generated voltage (24). The cation NMDG⁺ and the sugar sorbitol reduce the initial rate of L-[3H]aspartate uptake to ∼46 and ∼56%, respectively, but all of the K⁺ alternatives tested support uptake, suggesting not only that intracellular K⁺ is not required for the efficient functioning of GltPh, but that no cation is needed.

To confirm the lack of coupling of K⁺ to transport via GltPh, we examined the ability of K⁺ to induce the efflux of [3H]aspartate through the transporter. In these experiments, we diluted
GltPh-liposomes loaded with L-[3H]aspartate into various buffers and monitored the retained [3H]aspartate over time. This is an efflux experiment (also known as "reverse transport"); the outside buffer contains no Na⁺ or substrate so the outwardly directed Na⁺ gradient will drive the protein through its entire complement of states, resulting in the movement of one net aspartate out of the liposome. For an EAAT, K⁺ is essential in the outside buffer to observe efflux because the substrate-free transporter requires K⁺ countertransport to reorient (12, 33). Thus, if GltPh also requires K⁺ to reorient, we would expect that removal of this ion from the external buffer experiment would prevent efflux.

We examined efflux in GltPh to confirm that K⁺ is not required for reorientation of the aspartate-free transporter. In contrast to the EAATs, which display a strict requirement for K⁺ to observe efflux (31), we find that efflux can occur in the presence of a range of substances, including K⁺, C₇H₁₀, and sorbitol (Fig. 5C); these results further support the contention that K⁺ is not required for the reorientation of the aspartate-free transporter. The absence of efflux only in conditions where Na⁺ is equimolar on both sides of the liposome is unsurprising, because this condition eliminates the gradient that supplies the primary free energy to drive the transport cycle (Fig. 5C).

Temperature Dependence of Transport—Although our primary focus is to use GltPh as a model glutamate transporter, for which its properties at 20–30 °C are of most interest, analysis of the temperature dependence of its activity can yield insight into its mechanism as well as hints pertaining to its activity at its physiological temperature of >90 °C, a temperature where we cannot currently perform experiments. We measured initial rates of uptake as a function of temperature over a temperature range achievable in the laboratory, from near freezing to 40 °C (Fig. 6A). We find that above 40 °C our liposomes lose their integrity, and uptake measurements are not reproducible. Uptake is minimal at the low end of the measured temperature range with a roughly linear increase between 20° and 40 °C. Within the linear range the Q₁₀ is 3.7, consistent with a significant conformational change associated with transport. However, measurements of Na⁺ dependence of aspartate transport rates at 30 and 40 °C reveal minimal changes in Kᵢₘ value in this temperature range (Fig. 6B).

DISCUSSION

The crystal structure of a bacterial member of the glutamate transporter family has provided a plentitude of structural information about the broad family of glutamate transporters, yet we know little about the functional mechanisms of this prokaryotic transporter. Here we present a detailed functional characterization of GltPh, from P. horikoshii, where we explore the ion dependence of this aspartate transporter. Like the EAATs, aspartate transport via GltPh is coupled to the uptake of at least two Na⁺ ions, and in contrast to the EAATs, GltPh is not cou-
pled to the cotransport of H\(^+\) or the countertransport of K\(^+\) (Fig. 7, A and B).

Our results indicate that GltPh, reconstituted by incorporation into preformed vesicles, inserts into the membrane in both possible orientations in relatively equal proportions. Thus detailed kinetic analysis will be difficult in this system as transporters with different orientations will traverse the steps of the transport cycle in both possible directions, precluding clean separation of individual steps of the cycle. Because of this mixing of cycle directions, our measured kinetic parameters, \(K_m\), reflect lumped averages of the properties of both cycle directions. However, the bulk properties of the system, which depend only on the direction of the ionic gradients, can still be measured and permit the characterization of the ionic dependence of transport, as performed here. Based on our measured \(V_{\text{max}}\) values (Fig. 6B), we estimate the turnover number of the transporter at 0.29/min at 30 °C and 1.1/min at 40 °C. These are rough values, because they depend on a series of assumptions, including 100% efficiency of protein reconstitution, uniform size distribution of vesicles, and others. In addition, the transport rates at physiological temperatures for \textit{P. horikoshii} are probably much higher.

**FIGURE 5.** K\(^+\) dependence of Glt\(_{ph}\) transport. A, \(^{3}H\)-aspartate uptake when K\(^+\) (●) or Ch\(^+\) (■) is the cation in the inside buffer. Background uptake was measured in the absence of a Na\(^+\) gradient (▲). B, initial rates of \(^{3}H\)-aspartate uptake from similar experiments to those described in A with varying cations in the inside buffer. Initial rates were normalized to those measured in the presence of internal K\(^+\). The structure of the tetrakis cation is shown for reference. C, efflux experiments (solid lines) performed by diluting Glt\(_{ph}\)-liposomes containing \(^{3}H\)-aspartate into buffers containing 100 mM Na\(^+\) (●), 100 mM K\(^+\) (●), 100 mM Ch\(^+\) (△), or 200 mM sorbitol (△), normalized to the value at 5 s. The amount of \(^{3}H\)-aspartate retained in the liposomes is plotted as a function of time.

**FIGURE 6.** Temperature dependence of Glt\(_{ph}\) transport. A, initial rates of \(^{3}H\)-aspartate uptake (●) as a function of temperature. Background uptake is measured in protein-free liposomes (○). B, initial rates of \(^{3}H\)-aspartate uptake as a function of [Na\(^+\)] in the outside buffer at 30 °C (●; same as raw data from Fig. 3) and 40 °C (○). Data fit to the Hill equation yield \(K_m\) values of 2.2 ± 0.5 and 3.4 ± 0.4 mM, \(V_{\text{max}}\) of 6.5 ± 0.5 and 24.7 ± 0.9, and Hill coefficients of 2.5 ± 1.1 and 2.1 ± 0.5 at 30 and 40 °C, respectively.
The binding affinity and $K_m$ value are quite similar; this is consistent with the weaker apparent affinity for $Na^+$, suggesting that $Na^-$ off rates are faster, and binding of this ion is thus more likely to be in equilibrium and therefore obey Michaelis-Menten kinetics.

Glt$_{ph}$ demonstrates high selectivity for aspartate in contrast to properties observed in a report that describes Glt$_{ph}$ as a glutamate transporter driven by either a $Na^-$ or $H^+$ gradient (25). The glutamate transport we observe in the presence of a $Na^+$ or $H^+$ gradient is comparable with the levels measured by Raunser et al. (25); however, these levels are minimal compared with those of aspartate uptake, and large concentrations of glutamate are required to stimulate counterflow. We therefore conclude that Glt$_{ph}$ functions primarily as a $Na^+$-dependent aspartate transporter, without coupling to a proton gradient. This property contrasts with the EAATs, which transport L-aspartate and L-glutamate with similar micromolar affinities and which couple $H^+$ to the transport process.

Our data clearly demonstrate that at least two $Na^+$ ions are coupled to the uptake of each aspartate molecule and agree with binding data from Boudker et al. (14), demonstrating the coupling to two $Na^+$ ions to the binding of aspartate. These results do not rule out the possibility that three $Na^+$ ions could couple to transport, even though we measure a Hill coefficient of 2.4 - 2.6, leaving our measurement indeterminate in this regard. Similarly, the lack of a third cation site in the recent structure of Glt$_{ph}$ (14) does not rule out its presence as a slightly disordered binding site might prevent observation of its density, or the relatively poorly binding $Li^+$ used in the crystallographic experiments may not be able to displace this third $Na^+$ from its binding site. Of course, Hill coefficients can be influenced by other factors than the number of substrates binding to a protein. Ideally, an accurate stoichiometry would be measured by other thermodynamic methods, for example by determining reversal potentials of $Na^+$ and aspartate (3, 35). However, technical problems have hindered these measurements in our hands. Further experiments will be required to determine whether two or three $Na^+$ ions are coupled to aspartate transport via Glt$_{ph}$.

Experiments examining aspartate uptake with varying internal cations and aspartate efflux with varying external cations reveal that $K^+$ is not necessary for reorientation of the transporter. Even uncharged sorbitol supports uptake and efflux, although not quite at the levels of the tested inorganic cations, emphasizing the absence of a role for a cation in transporter reorientation.

These results demonstrate that, in contrast to their roles in the EAATs, $H^+$ and $K^+$ do not contribute to the Glt$_{ph}$ transport cycle, a result with major implications for understanding the roles of these ions in the transport mechanism of the broad glutamate transporter family. Furthermore, the absence of dependence of Glt$_{ph}$ on $K^+$ for transport and efflux reveals that this transporter can reorient in its empty (ion-free) form. This feature of the transport mechanism is strikingly different from the EAATs, which require $K^+$ countertransport to reorient the substrate-free protein. Interestingly, Glt$_{ph}$ lacks a glutamate residue at the position equivalent to one proposed to bind $K^+$ in GLT-1, the rat homolog of EAAT2 (Glu-404) (12). However,
Functional Analysis of Glt$_{ph}$

mutations of Glu-404 in GLT-1 seem to completely eliminate the ability of that protein to reorient along the K$^+$-dependent branch of the transport cycle, in contrast to Glt$_{ph}$, which can, of course, reorient in the absence of K$^+$. These mechanistic differences may prove useful in further probing the nature of the reorientation step. The same glutamate residue has also been proposed to bind the cotransported H$^+$ in EAAT3 (36). Given the pH independence of aspartate transport, and the absence of this residue in Glt$_{ph}$, our results are consistent with that proposed role.

Our temperature dependence data support the hypothesis that a substantial conformational change is associated with the Glt$_{ph}$ transport mechanism and suggest that transport rates at physiological temperatures for $P$. horikoshii may be substantially higher than those we observe. To measure transport rates at physiological temperatures would likely require the use of ether-linked lipids, prominent in hyperthermophiles, which might improve bilayer stability. Mechanistically, given that Na$^+$ seems to be governed by Michaelis-Menten kinetics, where $K_m = K_p$, the minimal variation of $K_m$ with temperature implies that changes in Na$^+$ binding are not responsible for the temperature dependence of transport rate, further supporting the hypothesis of a substantial conformational change accounting for the activation energy reported by $Q_{10}$

Taken together our data complete a framework to propose a simple kinetic model for Glt$_{ph}$ transport (Fig. 7C). Two to three Na$^+$ ions bind along with aspartate to the outward-facing transporter (states 4 $\rightarrow$ 1) facilitating its transition to an inward-facing, aspartate- and Na$^+$-bound state (2). Based on the crystal structure (14), which reveals one ion bound on the cytoplasmic (inward) side of the substrate and another bound on the extra-cellular (outward) side of the substrate, these binding steps are probably ordered as follows: with a first Na$^+$ binding followed by aspartate and then by a second Na$^+$. After Na$^+$ and aspartate dissociate on the cytoplasmic side of the membrane (3), the empty transporter can reorient to the outward-facing conformation (4) where it is again available to bind Na$^+$ and aspartate.

In a similar manner to the EAATs, uncoupled chloride movement through Glt$_{ph}$ is stimulated by the presence of Na$^+$ and substrate. However, Cl$^-$ movement has been observed in the absence of both Na$^+$ and aspartate (24). With the data currently available, it is difficult to assign Cl$^-$ flux to particular states in the transport cycle (Fig. 7C, gray arrow), but because both Na$^+$ and aspartate can activate Cl$^-$ permeability, we consider it likely that the states with these substrates bound favor the movement of Cl$^-$ through Glt$_{ph}$.

The characterization of Glt$_{ph}$ transport presented here establishes a functional basis for relating Glt$_{ph}$ structure to EAAT function and supports inferences of substantial conformational changes associated with alternating access models. We have established that the properties of Na$^+$-dependent aspartate transport and the uncoupled chloride flux are conserved between these evolutionarily distant members of the glutamate transporter family, whereas H$^+$ cotransport and K$^+$ countertransport are not. With these transport properties in hand, work can proceed toward explorations of the conformational changes underlying these transport phenomena.

Acknowledgments—We thank E. Gouaux (Vollum Institute and Howard Hughes Medical Institute, Oregon Health and Science University) for providing the Glt$_{ph}$ expression construct; Pat Curran for expert technical assistance; the members of the Mindell laboratory for challenging argumentation; and Kenton Swartz for critical readings of the manuscript.

REFERENCES

1. Danbolt, N. C. (2001) Prog. Neurobiol. 65, 1–105
2. Slotboom, D. J., Konings, W. N., and Lolkema, J. S. (1999) Microbiol. Mol. Biol. Rev. 63, 293–307
3. Zerangue, N., and Kavanaugh, M. P. (1996) Nature 383, 634–637
4. Billups, B., Rossi, D., and Attwell, D. (1996) J. Neurosci. 16, 6722–6731
5. Fairman, W. A., Vandenberg, R. J., Arzisa, J. L., Kavanaugh, M. P., and Amara, S. G. (1995) Nature 375, 599–603
6. Wadiche, J. I., Amara, S. G., and Kavanaugh, M. P. (1995) Neuron 15, 721–728
7. Wadiche, J. I., Arzisa, J. L., Amara, S. G., and Kavanaugh, M. P. (1995) Neuron 14, 1019–1027
8. Wadiche, J. I., and Kavanaugh, M. P. (1998) J. Neurosci. 18, 7650–7661
9. Yernool, D., Boudker, O., Jin, Y., and Gouaux, E. (2004) Nature 431, 811–818
10. Bendahan, A., Armon, A., Madani, N., Kavanaugh, M. P., and Kanner, B. I. (2000) J. Biol. Chem. 275, 37436–37442
11. Vandenberg, R. J., Arzisa, J. L., Amara, S. G., and Kavanaugh, M. P. (1995) J. Biol. Chem. 270, 17668–17671
12. Kavanaugh, M. P., Bendahan, A., Zerangue, N., Zhang, Y., and Kanner, B. I. (1997) J. Biol. Chem. 272, 1703–1708
13. Ryan, R. M., Mitrovic, A. D., and Vandenberg, R. J. (2004) J. Biol. Chem. 279, 20742–20751
14. Boudker, O., Ryan, R. M., Yernool, D., Shimamoto, K., and Gouaux, E. (2007) Nature 454, 387–393
15. Slotboom, D. J., Konings, W. N., and Lolkema, J. S. (2001) J. Biol. Chem. 276, 10775–10781
16. Slotboom, D. J., Sobczak, I., Konings, W. N., and Lolkema, J. S. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 14282–14287
17. Borre, L., Kavanaugh, M. P., and Kanner, B. I. (2002) J. Biol. Chem. 277, 13501–13507
18. Grunewald, M., Bendahan, A., and Kanner, B. I. (1998) Neuron 21, 623–632
19. Grunewald, M., and Kanner, B. I. (2000) J. Biol. Chem. 275, 9684–9689
20. Grunewald, M., Menaker, D., and Kanner, B. I. (2002) J. Biol. Chem. 277, 26074–26080
21. Leighton, B. H., Seal, R. P., Shimamoto, K., and Amara, S. G. (2002) J. Biol. Chem. 277, 29847–29855
22. Ryan, R. M., and Vandenberg, R. J. (2002) J. Biol. Chem. 277, 13494–13500
23. Seal, R. P., and Amara, S. G. (1998) Neuron 21, 1487–1498
24. Ryan, R. M., and Mindell, J. A. (2007) Nat. Struct. Mol. Biol. 14, 365–371
25. Rauiser, S., Appel, M., Ganea, C., Geldmacher-Kaufer, U., Fendler, K., and Kuhlbrandt, W. (2006) Biochemistry 45, 12796–12805
26. Gaillard, L., Slotboom, D. J., Knol, J., Lolkema, J. S., and Konings, W. N. (1996) Biochemistry 35, 6150–6156
27. Rigaud, J. L., Pitard, B., and Levy, D. (1995) Biochim. Biophys. Acta 1231, 223–246
28. Grewer, C., Balani, P., Weidenfeller, C., Bartusel, T., Tao, Z., and Rauen, T. (2005) Biochemistry 44, 11913–11923
29. Koch, H. P., and Larsson, H. P. (2005) J. Neurosci. 26, 1730–1736
30. Accardi, A., and Miller, C. (2004) J. Biol. Chem. 279, 18494–18499
31. Kenner, B. I., and Sharon, I. (1978) FEBS Lett. 94, 245–248
32. Kenner, B. I., and Sharon, I. (1978) Biochemistry 17, 3949–3953
33. Danbolt, N. C., Pines, G., and Kanner, B. I. (1990) Biochemistry 29, 6734–6740
34. Fersht, A. (1985) Enzyme Structure and Mechanism, 2nd Ed., pp. 101–103, W. H. Freeman & Co., New York
35. Graves, A. R., Curran, P. K., Smith, C. L., and Mindell, J. A. (2008) Nature 453, 788–792
36. Grewer, C., Watke, N., Rauen, T., and Bicho, A. (2003) J. Biol. Chem. 278, 2585–2592

17548 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 284 • NUMBER 26 • JUNE 26, 2009