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Engineering the Glutamate Transporter Homologue Glt_{Ph} Using Protein Semisynthesis

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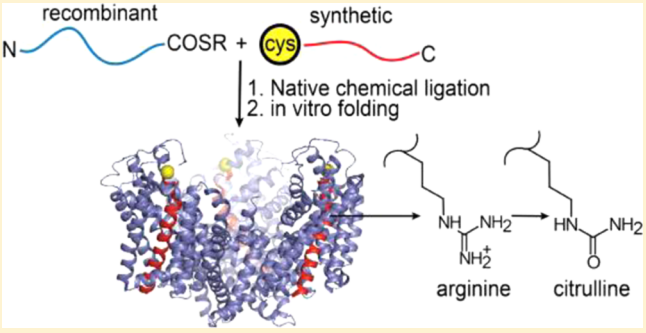
Supporting Information

ABSTRACT: Glutamate transporters catalyze the concentrative uptake of glutamate from synapses and are essential for normal synaptic function. Despite extensive investigations of glutamate transporters, the mechanisms underlying substrate recognition, ion selectivity, and the coupling of substrate and ion transport are not well-understood. Deciphering these mechanisms requires the ability to precisely engineer the transporter. In this study, we describe the semisynthesis of Glt_{Ph}, an archaeal homologue of glutamate transporters. Semisynthesis allows the precise engineering of Glt_{Ph} through the incorporation of unnatural amino acids and peptide backbone modifications. In the semisynthesis, the Glt_{Ph} polypeptide is initially assembled from a recombinantly expressed thioester peptide and a chemically synthesized peptide using the native chemical ligation reaction followed by in vitro folding to the native state. We have developed a robust procedure for the in vitro folding of Glt_{Ph}. Biochemical characterization of the semisynthetic Glt_{Ph} indicates that it is similar to the native transporter. We used semisynthesis to substitute Arg397, a highly conserved residue in the substrate binding site, with the unnatural analogue, citrulline. Our studies demonstrate that Arg397 is required for high-affinity substrate binding, and on the basis of our results, we propose that Arg397 is involved in a Na^+-dependent remodeling of the substrate binding site required for high-affinity Asp binding. We anticipate that the semisynthetic approach developed in this study will be extremely useful in investigating functional mechanisms in Glt_{Ph}. Further, the approach developed in this study should also be applicable to other membrane transport proteins.



Glutamate is the major excitatory neurotransmitter in the central nervous system.[1] Following release into the synaptic cleft during neurotransmission, glutamate is cleared by the actions of glutamate transporters that are also termed excitatory amino acid transporters (EAATs).[2,3] Glutamate transporters are present in plasma membranes of neuronal and glial cells and conduct the concentrative uptake of glutamate by coupling the transmembrane movement of glutamate to the cotransport of three Na^+ ions, one H^+ ion, and the countertransport of one K^+ ion.[4,5] Normal function of glutamate transporters is essential for maintaining the low extracellular concentration of glutamate that is important for efficient synaptic transmission and for preventing glutamate-induced neurotoxicity.[1,6]

Glutamate transporters are members of the solute carrier 1 (SLC1) family of secondary solute transporters that also includes a large number of prokaryotic and archaeal amino acid transporters.[1,7] Structural information about glutamate transporters is available from studies of the archaeal homologue Glt_{Ph} from *Pyrococcus horikoshii* (and the closely related Glt_{Tk} from *Thermococcus kodakarensis*).[8−12] Glt_{Ph} is a Na^+-coupled aspartate transporter.[9,13] Structural analysis has revealed that Glt_{Ph} is a homotrimer (Figure 1A). Each subunit consists of eight transmembrane helices (TM) and two re-entrant hairpin loops (HPs) that are arranged into two distinct domains: a central trimerization domain and a peripheral transport domain (Figure 1A,B). The binding sites for Asp and the sodium ions are contained within the transport domain.[9] The crystal structure shows an intricate network of interactions between the bound substrate and residues positioned at the tips of the hairpin loops, the highly conserved NMDGT sequence located in the unwound region of TM7, and polar residues on TM8 (Figure 1C). Transport of Asp in Glt_{Ph} is coupled to three Na^+ ions,[14,15] and the binding sites for two of the three Na^+ ions were visualized in the crystal structure (Figure 1C).[9] As observed in other secondary active transporters, the Na^+ binding sites in Glt_{Ph} show a heavy involvement of the backbone carbonyl oxygen atoms in coordinating the bound ions.[9]

The structural information presently available has set the stage for investigating the mechanisms underlying substrate recognition, ion selectivity, and the coupling of substrate and ion transport. These investigations require the ability to

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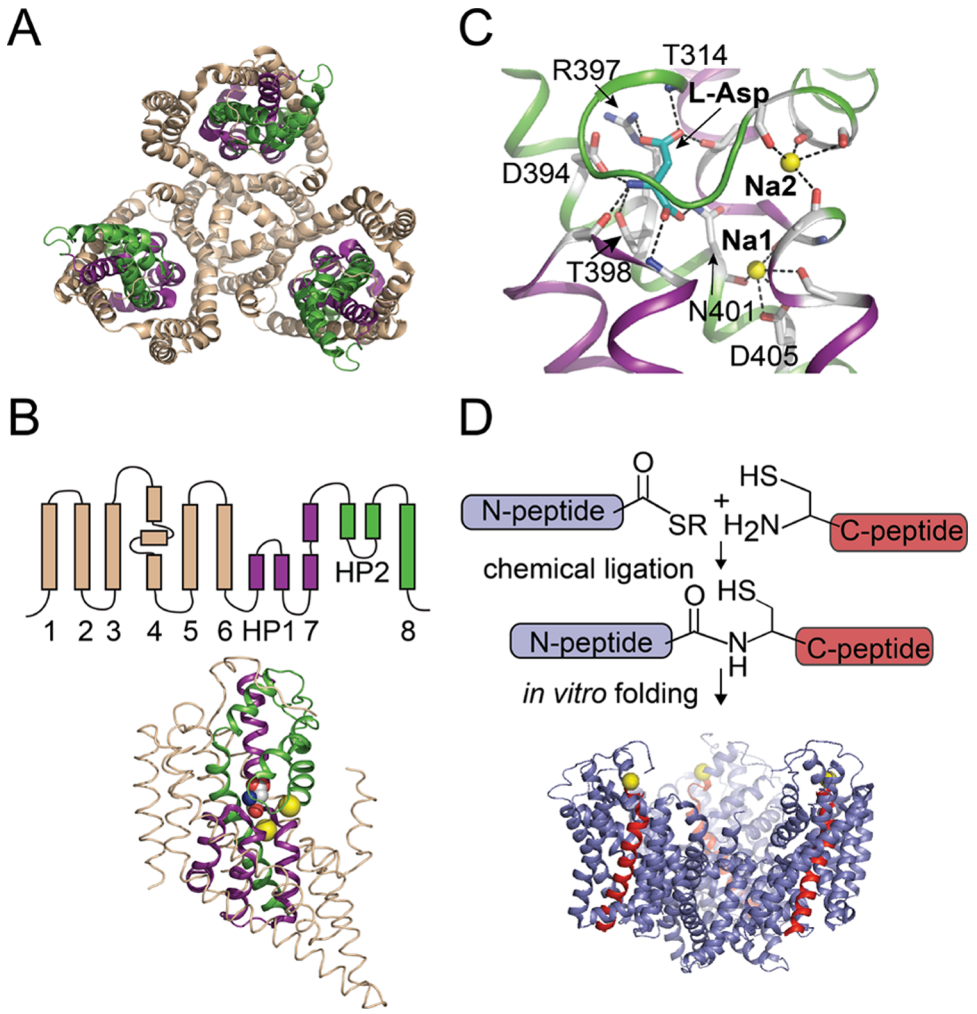

Figure 1. Structure of Glt_{Ph} and semisynthesis using native chemical ligation. (A) Top view of the trimeric Glt_{Ph} transporter (PDB entry 2nwx) showing the central trimerization domain (wheat) and the peripheral transport domain (green and purple). (B) Topology and structure of a single subunit of Glt_{Ph}. The regions of the subunit that contribute to the trimerization and the transport domain are colored as in panel A. Asp (space fill) and Na^+ ions (yellow spheres) bound to the transport domain are shown. (C) Close-up view of the Asp and Na^+ binding sites. Asp is shown as sticks, Na^+ ions are shown as yellow spheres. Interactions between the bound ligands and Glt_{Ph} are indicated by dashed lines. (D) Semisynthesis of Glt_{Ph} using native chemical ligation. The Glt_{Ph} polypeptide is assembled by the ligation reaction of a recombinantly expressed thioester peptide (N-peptide, residues 1−384, blue) and a synthetic peptide with an N-terminal Cys (C-peptide, residues 385−418, red). The ligation product is folded in vitro to the native state. The Cys residue at the ligation site, M385C, is represented as a yellow sphere.

precisely manipulate the interactions observed in the crystal structures between the protein and the bound substrate and ions. Conventional site-directed mutagenesis is of limited utility in this endeavor as it does not allow precise changes in the amino acid side chain or modifications of the protein backbone. Strategies for incorporating unnatural amino acids and protein backbone modifications will therefore be of great utility in these investigations.

Unnatural modifications of membrane proteins can be conducted by using nonsense suppression approaches.[16] There are two approaches presently being used. The first approach, which has been popularized by the Dougherty group, uses a suppressor t-RNA chemically acylated with the unnatural amino acid in conjunction with expression in *Xenopus* oocytes.[17] This approach is not suitable for Glt_{Ph} as it requires protein expression in oocytes. The other approach, pioneered by the Schultz group, uses an orthogonal suppressor t-RNA and synthetase pair that has been evolved for the unnatural amino acid.[18] This approach is presently limited in the types of unnatural amino acids that can be introduced. An alternate to these nonsense suppression approaches for incorporating unnatural modifications is to use chemical synthesis. Chemical synthesis is a very powerful method for protein modification as it allows the incorporation of a wide variety of unnatural amino acids and also allows modification of the protein backbone.[19] A key advantage of chemical synthesis over the nonsense suppression approaches is that it is not dependent on the ability of the ribosome to incorporate the modification and therefore provides greater freedom in the variety of modifications that can be introduced. With the goal of using chemical synthesis to modify Glt_{Ph}, we set out to develop the synthetic methodology required for Glt_{Ph}.

Chemical synthesis of a protein consists of peptide synthesis followed by in vitro folding to the native state. Peptide synthesis is conducted using solid phase peptide synthesis (SPPS) and is only efficient for peptides up to ∼50−60 amino acids in length.[19] As the Glt_{Ph} polypeptide is more than 400 amino acids in length, we used a semisynthetic approach in which SPPS is used for the region of interest, such as the Asp binding site, while the rest of the protein is obtained using

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recombinant means.[20] The synthetic peptide and the recombinant segment(s) are coupled using native chemical ligation (NCL) to form the Glt_{Ph} polypeptide (Figure 1D). In NCL, a peptide with a C-terminal thioester reacts with a peptide with an N-terminal Cys, linking the peptides with a native peptide bond at the ligation site.[20,21] A critical step in the synthesis is the in vitro folding process required to convert the synthetic polypeptide to the native state. Glt_{Ph} presents a substantial challenge for in vitro folding as it is a multimeric, multidomain membrane protein.[22]

In this study, we describe the semisynthesis of Glt_{Ph}. As required for the semisynthesis, we develop an efficient procedure for the in vitro folding of Glt_{Ph}. We use semisynthesis to investigate the role of Arg397, a conserved Arg in the substrate binding site. Our results demonstrate that the Arg397 residue is required for high-affinity substrate binding, and on the basis of our results, we propose that Arg397 is involved in a Na^+-dependent remodeling of the substrate binding site required for high-affinity Asp binding.

EXPERIMENTAL PROCEDURES

Native Expression and Purification of Glt_{Ph}. Glt_{Ph} constructs used in this study carried an N-terminal His_6 tag and a C-terminal Strep tag (WSHPQFEK). The Glt_{Ph} constructs were subcloned into the pBCH/G4 vector (kindly provided by E. Gouaux) and expressed in *Escherichia coli* TOP10 cells (Life Technologies). Protein expression was induced with 0.1% (w/v) arabinose at an optical density of 0.8−1.0 for 4 h at 37 °C. Following expression, cells were pelleted and suspended in 20 mM HEPES-NaOH (pH 7.5) and 200 mM NaCl, and membranes were prepared as described previously.[23] Membranes were solubilized in dodecyl β-D-maltopyranoside [DDM, 2% (w/v)], and the Glt_{Ph} protein was purified using Ni-NTA resin (Qiagen), followed by size exclusion chromatography (SEC). SEC was conducted using a Superdex S200 column (GE Biosciences) using 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM glutamate, and 0.1% (w/v) DDM as the column buffer.

Unfolding and Refolding of the Glt_{Ph} Transporter. Unfolding of Glt_{Ph} was conducted as described for the K_vAP channel.[23] Briefly, Triton X-100 (Tx-100) was added to the Glt_{Ph} solution to a concentration of 2% (v/v), and the protein was precipitated by the addition of 15% (w/v) trichloroacetic acid (TCA) and incubation at 4 °C for 30 min. The protein precipitate was collected by centrifugation, washed twice with acetone and 0.1% TFA (trifluoroacetic acid), and then solubilized in 50% TFE (trifluoroethanol) and 0.1% TFA. The TFE solution was lyophilized to provide the unfolded Glt_{Ph} polypeptide that was used for the refolding studies.

The lipid vesicles used for refolding Glt_{Ph} were prepared from Soy Total Lipid Extract (Asolectin, Avanti Polar Lipids). For vesicle formation, lipids were dissolved in cyclohexane and lyophilized. The lyophilized lipids were hydrated for 1 h at a concentration of 20 mg/mL in 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl, 10 mM DTT, and 1 mM glutamate and then sonicated to yield the lipid vesicles.

For refolding, the unfolded polypeptide was dissolved in 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl, 1% SDS, 10 mM DTT, and 1 mM glutamate, then diluted 10-fold into the lipid vesicle solution, and briefly sonicated. Refolding was allowed to proceed for 3−4 h at room temperature, and then the samples were dialyzed against 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl, 0.5 mM DTT, and 1 mM glutamate. Regenerated cellulose membranes with a 6000−8000 molecular weight cutoff were used for dialysis. Refolded proteins were solubilized and purified as described for the native Glt_{Ph}.

Cross-Linking of Glt_{Ph}. Assessment of the multimeric nature of Glt_{Ph} was achieved by chemical cross-linking using 0.25% (w/v) glutaraldehyde for 10 min at room temperature. The cross-linking reaction was quenched by the addition of 100 mM Tris. The cross-linked products were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and visualized by staining with Coomassie Blue.

Aspartate Binding Assay. Binding assays were conducted on Glt_{Ph} transporters with the L130W substitution as previously described.[9] Briefly, purified Glt_{Ph} was dialyzed (thrice) against 200 volumes of the assay buffer [20 mM HEPES/Tris buffer (pH 7.5) containing 200 mM choline chloride, 1 mM NaCl, and 0.1% DDM] to ensure that the transporters were devoid of Asp. The binding assays were conducted using ∼100 nM Glt_{Ph}, and binding of Asp was monitored by the change in Trp fluorescence with excitation at 295 nm and emission monitored at 334 nm. The changes in fluorescence were normalized to the initial fluorescence, and ligand binding curves were fit to the equation $F_{bound} = ([Asp]/K_d)/(1 + [Asp]/K_d)$ to determine the K_d for Asp. When the K_d for Asp was comparable to the protein concentration, the equation $F_{bound} = \{K_d + [P] + [S] - [(K_d + [P] + [S])^2 - 4[P][S]]^{1/2}\}/(2[P])$ was used.[9]

Aspartate Transport Assays. The Glt_{Ph} transporter was reconstituted into liposomes as previously described, and the proteoliposomes obtained were snap-frozen in liquid N_2 and stored at −80 °C.[9,13] Previously frozen proteoliposomes were thawed and centrifuged at 265000*g* for 70 min. Pelleted proteoliposomes were resuspended in 100 K buffer [20 mM HEPES-KOH (pH 7.5) and 100 mM KCl] at 5 mg/mL lipid, subjected to two freeze−thaw cycles with liquid N_2, and extruded through 400 nm filters. Extruded proteoliposomes were centrifuged and resuspended in 100 K buffer at 333 mg/mL lipid. The uptake reaction was initiated by diluting the proteoliposomes 133-fold into the reaction buffer [20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl, and 100 nM [^{14}C]Asp] at room temperature. For each time point, a 250 μL aliquot was removed and diluted 10-fold into ice-cold quench buffer [20 mM HEPES-KOH (pH 7.5) and 100 mM KCl] followed by filtration over nitrocellulose filters (0.22 μm, Millipore). Filters were washed twice with 2 mL of ice-cold quench buffer and assayed for radioactivity. Background levels of [^{14}C]Asp uptake were determined in the absence of sodium (100 K buffer on both sides). The inhibition experiments were performed by first incubating proteoliposomes in buffer [20 mM HEPES-NaOH (pH 7.5) and 200 mM NaCl] containing 10 μM TBOA (Tocris Bioscience) for 5 min, following addition of 100 nM [^{14}C]Asp. Uptake data are fit to single exponentials for presentation.

Recombinant Expression of Glt_{Ph} (1−384) Thioester. A sandwich fusion strategy was used for expression of the Glt_{Ph} 1−384 (N-peptide) thioester.[24] The fusion protein consisted of Glt_{Ph} residues 1−384 sandwiched between glutathione *S*-transferase (GST) at the N-terminus and the gyrA intein−chitin binding domain at the C-terminus. A thrombin site, a His_6 tag, and a factor Xa site were present between GST and the Glt_{Ph} sequence. Expression of the sandwich fusion in inclusion bodies was conducted in *E. coli* Rosetta2 (DE3) cells (Merck) using the autoinduction protocol.[23,25] For isolation of the inclusion bodies, cells were pelleted and resuspended in 20

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mM Tris-HCl (pH 7.5), 0.2 M NaCl, 1 mM $MgCl_2$, DNase (5 μg/mL), lysozyme (0.1 mg/mL), and 1 mM phenylmethanesulfonyl fluoride. The cells were incubated at room temperature while being gently stirred for 30 min and then lysed by sonication. Tx-100 was added (1%), and the cell lysate was stirred at room temperature for 30 min. The soluble and insoluble fractions were separated by centrifugation at 12000*g* for 10 min. The insoluble fraction, which contains the inclusion bodies, was washed twice with 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1% Tx-100. The inclusion bodies were solubilized in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1% (w/v) *N*-lauryl sarcosine (NLS) and digested with thrombin (Roche, 1 unit/L of culture) overnight, to cleave the Glt_{Ph}–intein fusion from GST.

For purification of the Glt_{Ph}–intein fusion, the thrombin cleavage mixture was diluted with an equal volume of 20 mM Tris-HCl (pH 7.5) and 200 mM NaCl. Tx-100 was added to a final concentration of 2%, and the Glt_{Ph}–intein fusion was purified using metal affinity chromatography (Talon, Clontech). Following purification, the Glt_{Ph}–intein fusion was dialyzed overnight against 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1% Tx-100 and then cleaved by the addition of 2-mercaptoethanesulfonic acid (MESNA, 0.15 M) to generate the Glt_{Ph} thioester peptide. Typically, ~50% cleavage of the intein fusion was observed following incubation with MESNA for 1–2 days at room temperature. The thiolysis mixture was precipitated using TCA and lyophilized using a procedure similar to that used for unfolding of Glt_{Ph}.

Chemical Synthesis of the Glt_{Ph} C-Terminal Peptides. The C-peptide, Glt_{Ph} residues 385–418 with a C-terminal Strep tag (in italics) (CILGIDAILDMG**R**TMVNVTGDLTGTAIVAKTEGT*GSGWSHPQFEK*), was synthesized on a PAM (phenylacetamidomethyl) resin using a slightly modified version of the in situ neutralization/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation protocol for Boc solid phase peptide synthesis.[26] The β-branched amino acids in the sequence were double-coupled using HBTU in DMSO to ensure complete coupling.[27] Following chain assembly, global deprotection and cleavage from the solid phase were conducted using anhydrous hydrofluoric acid (HF). The crude material obtained after HF cleavage was solubilized in 50% buffer B (9:1 acetonitrile:H_2O + 0.1% TFA), purified by RP-HPLC on a preparative C4 column using a 30 to 70% gradient of buffer B, and confirmed by electrospray mass spectrometry [observed mass for the wild-type peptide of 4691.6 ± 0.1 Da (standard deviation), calculated mass of 4692.3 Da (Figure 1A,B of the Supporting Information)]. To generate the R397 citrulline (Cit) mutant, a C-peptide with the Arg underlined substituted with Cit was synthesized. The R397 Cit peptide was synthesized and purified as described for the wild-type peptide [observed mass for the R397 Cit peptide of 4693 ± 0.8 Da, calculated mass of 4693.3 Da (Figure 1C,D of the Supporting Information)].

Assembly of the Semisynthetic Glt_{Ph} Transporters. The ligation reaction between the recombinant Glt_{Ph} N-peptide thioester and the synthetic C peptide was conducted in 0.1 M phosphate buffer (pH 8.0) and 1% (w/v) dodecylphosphocholine (Fos-12). A ~5–10-fold molar excess of the C-peptide was used to drive the ligation reaction to completion. The reaction was initiated by the addition of thiophenol [2% (v/v)] and conducted at room temperature with gentle stirring. The reaction was monitored by SDS–PAGE and was typically complete after 12–24 h. The ligation reaction was terminated by the addition of DTT (0.1 M, 30 min at room temperature), and the mixture was diluted 10-fold with 0.1 M phosphate buffer (pH 8.0), 300 mM NaCl, and 0.1% DDM and purified using Streptactin resin (IBA Lifesciences). The purified ligation product was precipitated with TCA, lyophilized, and folded in vitro as described above for the native Glt_{Ph}.

RESULTS

In Vitro Folding of Glt_{Ph}. A key requirement for our synthetic strategy is the ability to conduct in vitro folding of Glt_{Ph}. As in vitro folding of Glt_{Ph} has not been previously demonstrated, we initially investigated the feasibility of this step. For these studies, we used the Glt_{Ph} polypeptide obtained by unfolding the native protein. To ensure extensive unfolding, Glt_{Ph} was precipitated with TCA and acetone, dissolved in TFE and buffer A [H_2O and 0.1% (v/v) TFA], lyophilized, and then solubilized in 1% SDS. Unfolding of Glt_{Ph} using this protocol was confirmed by glutaraldehyde cross-linking. Native Glt_{Ph} is a trimer, and glutaraldehyde cross-linking of the native transporter gives a protein band that migrates in a manner

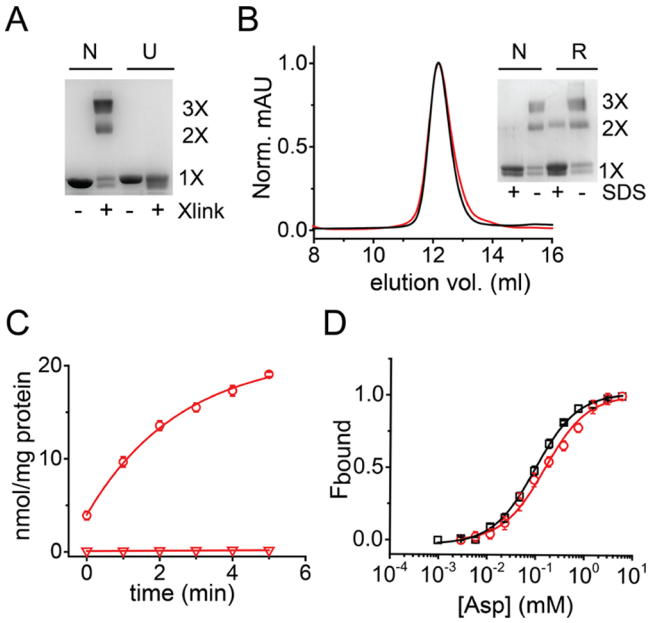

Figure 2. In vitro folding of Glt_{Ph}. (A) SDS–PAGE gel showing the native (N) and unfolded (U) Glt_{Ph} with (+) and without (−) glutaraldehyde cross-linking. Native Glt_{Ph} cross-links to a trimer, while the unfolded protein is monomeric. (B) Size exclusion chromatography showing a similar elution profile for native (black) and refolded (red) Glt_{Ph}. The inset shows glutaraldehyde cross-linking of the peak fraction for native and refolded Glt_{Ph} before (−) and after (+) treatment with 1% SDS. In panels A and B, the oligomeric nature of the cross-linked band (1×, 2×, and 3×) is indicated. (C) Uptake of Asp by refolded Glt_{Ph}. Time course of uptake of [^{14}C]Asp into vesicles containing refolded Glt_{Ph} in the presence of a Na^+ gradient (circles; n = 3). Uptake of [^{14}C]Asp is not observed in the absence of a Na^+ gradient [100 mM K^+ on both sides of the membrane (triangles)]. (D) Asp binding by native and refolded Glt_{Ph}. The fraction of the protein bound (F_{bound}) was determined by dividing the change in fluorescence upon addition of Asp by the total change at the end of the titration. Solid lines are fits to the data using the equation described in Experimental Procedures with K_d values of 97 ± 4.8 μM (n = 7) for native Glt_{Ph} (black) and 154 ± 19 μM (n = 6) for refolded Glt_{Ph} (red). The binding assays were conducted in 1 mM Na^+. Error bars correspond to the standard error of the mean.

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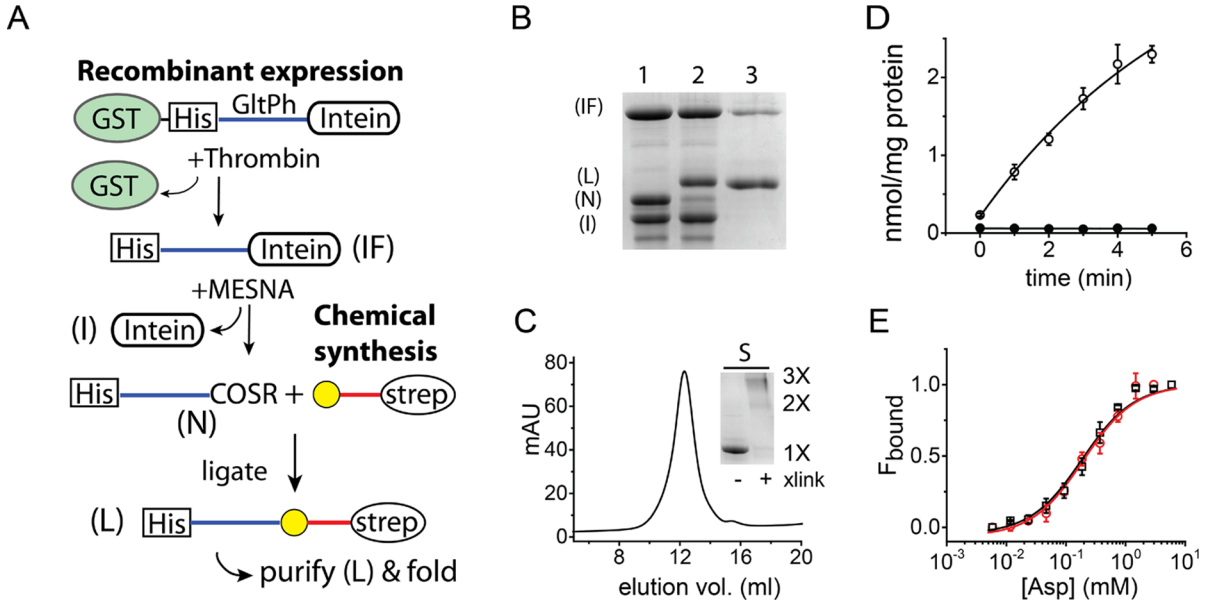

Figure 3. Semisynthesis of Glt_{Ph}. (A) Strategy for the semisynthesis of Glt_{Ph}. Glt_{Ph} residues 1−384 are sandwiched between glutathione *S*-transferase (GST) and the gyrA intein (I). A thrombin cleavage site and His_6 tag are present between GST and the Glt_{Ph} sequence. Proteolysis with thrombin releases the Glt_{Ph}−intein fusion (IF), which is purified using the His_6 tag and cleaved with MESNA to provide the N-peptide thioester (N). The N-peptide thioester is ligated to a synthetic C-peptide (residues 385−418) with an N-terminal Cys (yellow sphere) and a C-terminal Strep tag. The ligation reaction yields the full length Glt_{Ph} polypeptide (L), which is purified using the Strep tag and then folded in vitro to the native state. (B) SDS−PAGE gel detailing the assembly and purification of the semisynthetic Glt_{Ph} polypeptide: lane 1, treatment of the Glt_{Ph}−intein fusion (IF) with MESNA that cleaves the N-peptide thioester (N) from the intein (I); lane 2, NCL of the N-peptide thioester with the synthetic C-peptide that yields the semisynthetic Glt_{Ph} polypeptide (L); lane 3, semisynthetic Glt_{Ph} polypeptide following purification using the C-terminal Strep tag. (C) Size exclusion chromatography of semsiysnthetic Glt_{Ph}. The inset shows an SDS−PAGE gel of the semisynthetic Glt_{Ph} with (+) and without (−) glutaraldehdye cross-linking. (D) Uptake of Asp by the semisynthetic Glt_{Ph}. Time course of uptake of [^{14}C]Asp into vesicles containing the semisynthetic Glt_{Ph} in the presence (○; $n = 3$) and absence of a Na^+ gradient (●; $n = 3$). (E) Asp binding by semisynthetic Glt_{Ph}. Asp binding assays as described in Figure 2D for the semisynthetic (red circles; $K_d = 229 \pm 28$ μM; $n = 3$) and the native control, M385C Glt_{Ph} (black squares; $K_d = 210 \pm 21$ μM; $n = 3$).

corresponding to a trimer on SDS−PAGE (Figure 2A). Unfolding of Glt_{Ph} results in a loss of the trimer, and glutaraldehdye cross-linking of the unfolded protein gives only a protein band corresponding to the monomer on SDS−PAGE (Figure 2A).

Guided by our previous success in using lipid vesicles for in vitro folding of ion channels,[23,28] we investigated whether a similar approach could be used for refolding Glt_{Ph}. We used glutaraldehyde cross-linking to check for refolding. We observed that upon dilution of unfolded Glt_{Ph} into lipid vesicles, glutaraldehyde cross-linking gave a protein band corresponding to a trimer suggesting refolding of Glt_{Ph}. We purified refolded Glt_{Ph} by using metal affinity chromatography followed by size exclusion chromatography (SEC). The SEC elution profile of the refolded protein was similar to that of native Glt_{Ph}, and glutaraldehyde cross-linking confirmed the trimeric nature of refolded Glt_{Ph} (Figure 2B).

We used substrate binding and Asp transport assays to examine the functionality of refolded Glt_{Ph}. We reconstituted the purified refolded Glt_{Ph} into lipid vesicles for the measurement of transport activity. We observed robust uptake of [^{14}C]Asp in the presence of an inwardly directed Na^+ gradient in proteoliposomes containing the refolded transporter (Figure 2C). No uptake activity was observed in the absence of a Na^+ gradient (100 mM K^+ on both sides of the membrane). The specific activity of transport measured for the refolded protein was comparable to specific activity values reported for native Glt_{Ph}.[9,13,29,30] Uptake of Asp in Glt_{Ph} is blocked by TBOA (DL-threo-β-benzyloxyaspartic acid),[9,13] and we observed a similar extent of inhibition by TBOA for native and refolded Glt_{Ph} [31 ± 10% for the native form compared to 40 ± 1% for refolded Glt_{Ph} ($n = 3$)]. To investigate substrate binding, we used a fluorescence-based assay that relies on a Trp substitution at position 130 (L130W) and reports on the coupled binding of Asp and Na^+.[9] Using this assay, we measured similar K_d values for binding of Asp to native and refolded L130W Glt_{Ph} (Figure 2D). These similar biochemical and functional properties indicate that refolded Glt_{Ph} is similar to the native transporter.

Semisynthesis of Glt_{Ph}. In the semisynthesis of Glt_{Ph}, we focused on TM8, which contains residues implicated in both substrate and cation binding.[8] We selected Met385, a residue upstream of TM8, as the ligation site because Asp transport assays indicated that a Cys substitution at this site, which is required for the ligation chemistry, is well-tolerated. The semisynthesis therefore calls for the recombinant generation of a thioester polypeptide corresponding to residues 1−384 of Glt_{Ph} (N-peptide) and a synthetic fragment corresponding to residues 385−418 with an N-terminal Cys (C-peptide) (Figure 3A; see also Figure 1D). To test the synthetic strategy, we initially assembled semisynthetic Glt_{Ph} with the wild-type sequence. The WT synthetic C-peptide was obtained by SPPS and purified using RP-HPLC in good yields (Figure 1A,B of the Supporting Information). We introduced a Strep tag at the C-terminus of the synthetic peptide for ease of purification of the ligation product.[31] To generate the recombinant N-peptide thioester, we used the sandwich fusion approach that we have previously described.[24] In this approach, the gyrA

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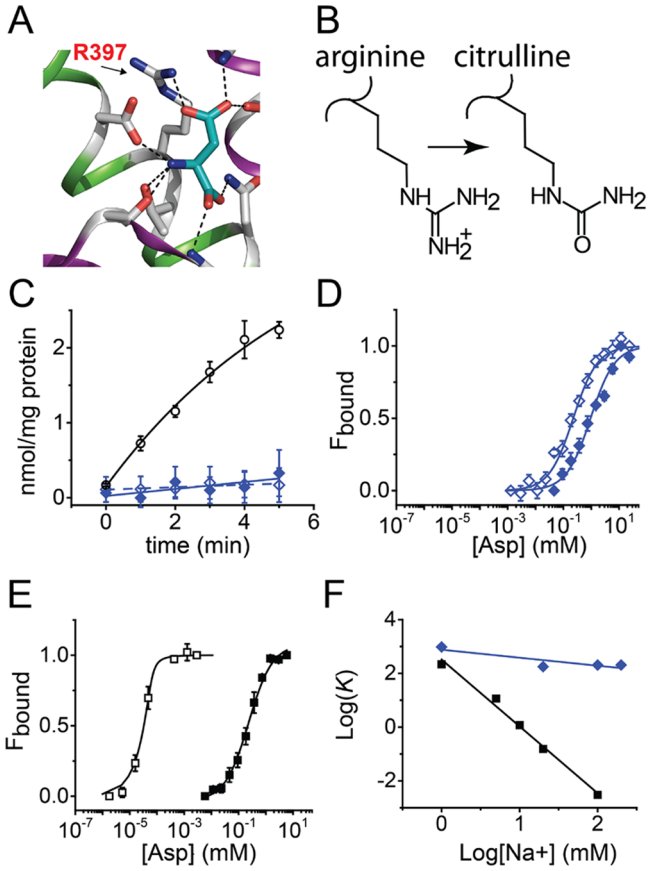

Figure 4. Arg397 in the substrate binding site of Glt_{Ph}. (A) Close-up view of the Asp binding site highlighting the interaction of Arg397 with the β-carboxyl group of the bound Asp. (B) Structures of the side chains of Arg and the unnatural amino acid citrulline (Cit). (C) Time course for uptake of [^{14}C]Asp into vesicles containing R397 Cit Glt_{Ph} in the presence 0.1 μM [^{14}C]Asp (empty diamonds; n = 2) and 1.0 μM [^{14}C]Asp (filled diamonds; n = 3). For comparison, the time course for uptake of [^{14}C]Asp into vesicles containing semisynthetic WT Glt_{Ph} in the presence of 0.1 μM [^{14}C]Asp (empty circles) is also shown (data from Figure 3D). For the sake of clarity, data have been corrected for background uptake determined in the absence of a Na^+ gradient (100 mM K^+ on both sides of the membrane). (D and E) Asp binding to R397 Cit and the WT, respectively, at 1 mM (filled symbols) and 100 mM Na^+ (empty symbols) conducted as described in the legend of Figure 2D. A shift from 1 to 100 mM Na^+ decreases the K_d for binding of Asp to the native control (M385C Glt_{Ph}) from 210 ± 21 μM (n = 3) to 3.01 ± 0.6 nM (n = 4), while only a modest decrease [from 961 ± 102 μM (n = 3) to 204 ± 15 μM (n = 4)] is observed for R397 Cit Glt_{Ph}. (F) Logarithmic plots of Asp K_d values (micromolar) vs log[Na^+] (millimolar) for the native control (black squares) and R397 Cit Glt_{Ph} (blue diamonds).

intein is fused to residues 1−384 of Glt_{Ph} for the introduction of the C-terminal thioester while GST is appended to the N-terminus. The N-terminal GST directs expression of the fusion to inclusion bodies, thereby preventing cell lethality caused by expression of the gyrA intein fused to a transmembrane segment.[27] Following expression, the inclusion bodies were isolated, the GST tag was removed by proteolysis, and the N-peptide−intein fusion was purified. The Glt_{Ph} N-peptide thioester was then generated by thiolysis of the intein fusion (Figure 3A,B).

Our attempts to purify the thioester polypeptide using RP-HPLC were not successful likely because of the size and hydrophobic nature of the polypeptide, so we conducted the ligation reaction with the C-peptide without purification. Prior to ligation, the thiolysis mixture was precipitated with TCA and acetone and colyophilized with the C-peptide. The ligation reaction was conducted in the presence of dodecylphosphocholine (Fos-12) to keep the reactants soluble during the course of the reaction. We observed that the ligation reaction proceeded to ∼90% completion after a 12−24 h incubation at room temperature (Figure 3B).

Following ligation, the semisynthetic Glt_{Ph} polypeptide (and the unreacted C-peptide) was separated from the unreacted N-peptide thioester and the uncleaved N-peptide−intein fusion by using the Strep tag present at the C-terminus (Figure 3A,B). Following Strep tag purification, further separation of the Glt_{Ph} polypeptide from the unreacted C-peptide can be conducted using the His_6 tag present at the N-terminus of the ligation product. However, the C-peptide did not interfere with the folding of Glt_{Ph}, so the folding reaction was conducted without further purification. In vitro folding of the semisynthetic Glt_{Ph} was conducted using the lipid-based protocol that we identified using the unfolded Glt_{Ph}.

The semisynthetic Glt_{Ph} transporter after in vitro folding was purified in a manner similar to that used for refolded Glt_{Ph}. The semisynthetic Glt_{Ph} had a retention time on SEC similar to that of native Glt_{Ph}, and glutaraldehyde cross-linking confirmed the trimeric nature of the semisynthetic transporter (Figure 3C). We reconstituted the purified semisynthetic Glt_{Ph} transporter into lipid vesicles for measurement of Asp uptake. Transport assays showed uptake of [^{14}C]Asp in the presence of an inwardly directed Na^+ gradient, while no uptake activity was observed with 100 mM K^+ on both sides of the membrane, indicating the semisynthetic Glt_{Ph} is functional (Figure 3D). The specific activity of transport measured for semisynthetic Glt_{Ph} was lower than that of native Glt_{Ph} but was still within the range of values reported in the literature for the native transporter.[9,13,29,30] Further, Asp uptake observed for semisynthetic Glt_{Ph} was inhibited by TBOA to an extent similar to that observed for native Glt_{Ph} [42 ± 4% for semisynthetic compared to 31 ± 10% for native Glt_{Ph} (n = 3)]. We also assembled a semisynthetic Glt_{Ph} with the L130W substitution to assay for Asp binding. These assays indicated that the K_d value for binding of Asp to the semisynthetic Glt_{Ph} was similar to that of the native control [M385C Glt_{Ph} (Figure 3E)]. Taken together, these assays indicate the functional similarity of semisynthetic Glt_{Ph} to native Glt_{Ph}. The limiting component in the semisynthesis of Glt_{Ph} is the N-peptide thioester. Starting with the thioester obtained from 8 L of culture, we were able to obtain ∼0.2−0.3 mg of purified semisynthetic Glt_{Ph}.

Arg397 in the Asp Binding Site. The substrate binding site in Glt_{Ph} contains an Arg residue, Arg397, that forms a salt bridge with the β-carboxyl group of the bound Asp (Figure 4A). Arg397 is proposed to play a key role in determining amino acid selectivity as it is highly conserved in EAATs and the prokaryotic acidic amino acid transporters but is substituted with a Thr or Cys in the neutral amino acid transporters.[9,32] Further, substitution of the equivalent Arg in mammalian glutamate transporters with a neutral or negatively charged amino acid abolished glutamate uptake but did not affect the interaction of neutral amino acids with the transporter.[33−35] To further investigate the role of Arg397 in Glt_{Ph}, we used semisynthesis to replace Arg397 with the unnatural amino acid Cit. Cit is isosteric to Arg but lacks a positive charge on the side chain (Figure 4B). The Arg to Cit substitution therefore

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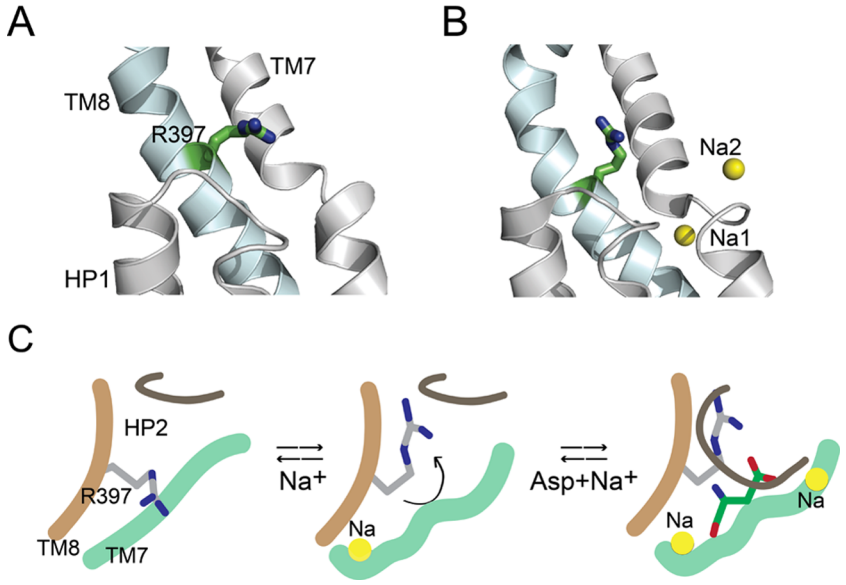

Figure 5. Ion-induced conformational change in the substrate binding site of Glt_{Ph}. (A and B) Crystal structures of the inward-facing conformation of Glt_{Ph} in the apo state (PDB entry 4p19) and in the presence of Tl^+ (PDB entry 4p6h), respectively. Close-up view of the substrate binding site showing HP1 and TM7 (gray) and TM8 (blue). Arg397 is shown as sticks. The cation binding sites, Na1 and Na2, are represented as yellow spheres. (C) Model depicting a proposed role for Arg397. Binding of a Na^+ ion causes a conformational change in the substrate binding site that reorients Arg397 to create a high-affinity Asp binding site. Binding of Asp is followed by the binding of another Na^+ ion that is concomitant with the closure of HP2 leading to transport. Only the sodium sites visualized in the Glt_{Ph} crystal structures[9] are shown in the model.

minimally perturbs the substrate binding site and specifically tests the role of a positively charged side chain in substrate binding and transport. To substitute Arg397 with Cit, we synthesized a C-peptide with the R397 to Cit substitution and used this peptide to assemble a semisynthetic R397 Cit Glt_{Ph} (Figure 1C,D of the Supporting Information).

We purified semisynthetic R397 Cit Glt_{Ph} and reconstituted it into lipid vesicles to measure Asp uptake. We did not observe Asp uptake in the Cit mutant under the standard assay conditions (0.1 μM Asp) or upon using a 10-fold higher concentration of Asp (Figure 4C). The lack of uptake at the Asp concentrations tested led us to investigate whether the compromised uptake in the Cit mutant was due to perturbed binding of Asp. We used semisynthesis to generate R397 Cit Glt_{Ph} with the L130W substitution for binding assays. We conducted Asp binding assays over a range of Na^+ concentrations from 1 to 100 mM. Surprisingly, we observed that the effect of the Cit substitution on Asp binding was dependent on the Na^+ concentration. At 1 mM Na^+, the K_d for binding of Asp to the Cit mutant (961 ± 102 μM) was only slightly altered compared to that of the native control, M385C Glt_{Ph} [210 ± 21 μM (Figure 4D,E)]. However, at 100 mM Na^+, there was an ~50000-fold difference between the K_d of the Cit mutant (204 ± 15 μM) and that of M385C Glt_{Ph} (3.01 ± 0.6 nM). The Asp binding assays at different Na^+ concentrations show that the removal of the positive charge has a minimal effect on substrate binding at low Na^+ concentrations but a substantial effect at high Na^+ concentrations. In wild-type Glt_{Ph}, the binding of Asp is coupled to the binding of Na^+. A plot of log K_d for Asp versus log[Na^+] yields a straight line with a slope of −2.48, indicating that the binding of Asp is coupled to the binding of at least two Na^+ ions (Figure 4F).[9] In the Cit mutant, the plot had a slope of −0.30, indicating a lack of coupling between Asp and Na^+ binding in this mutant (Figure 4F).

DISCUSSION

In this study, we developed a semisynthesis for Glt_{Ph}. Glt_{Ph} is, to the best of our knowledge, the largest membrane protein to be obtained using synthetic or semisynthetic means. A critical step in the semisynthesis was in vitro folding, and we have developed a robust procedure using lipid vesicles for the in vitro folding of Glt_{Ph}. The successful in vitro folding of Glt_{Ph} demonstrates that integral membrane proteins with a complex subunit topology can be folded in vitro.

The semisynthetic strategy that we developed is for TM8 but can be easily extended to other functionally important regions such as TM7 or the hairpin regions by selecting appropriate ligation sites. The yields of the semisynthesis presently are sufficient for functional and biochemical studies but are not yet sufficient for structural studies. The limiting step in the current strategy is the generation of the N-peptide thioester. We envision that by exploring various inteins, we will be able to identify an intein that is more efficient than the gyrA intein presently used, to improve the yields of the thioester peptide and thereby the yields of semisynthetic Glt_{Ph}.

We used semisynthesis to replace Arg397 in the substrate binding site with Cit. Cit is isosteric to Arg but neutral and thereby provides us with a precise means of evaluating the role of the positively charged residue in the substrate binding site. Comparison of binding of Asp to the wild type and the Cit mutant showed at low Na^+ concentrations, substituting the Arg side chain had only a small (4.5-fold) effect on Asp binding compared to a substantial (~50000-fold) effect at high Na^+ concentrations. This difference in the effect of substituting the Arg side chain (on Asp binding) with Na^+ concentration indicates that R397 is critical for high-affinity Na^+-coupled Asp binding. The coupling between Na^+ and Asp binding in Glt_{Ph} has been proposed to involve conformational changes in the substrate binding site.[12] As coupling of Na^+ to Asp binding is lost upon substitution of Arg397, we speculate that the conformational change in the substrate binding site involves

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Arg397. A precedent for a conformational change in Arg397 on ion binding is observed upon comparison of the inward-facing structure of Glt_{Ph} in the apo state to the inward-facing structure in the Tl^+-bound state (Figure 5A,B).[12] A similar repositioning of the equivalent Arg side chain is observed upon comparison of the outward-facing apo structure of Glt_{Tk} to the outward-facing Na^+- and Asp-bound structure of Glt_{Ph}.[9,10]

Studies of EAATs indicate an ordered binding process in which two Na^+ ions bind prior to glutamate, followed by the binding of the third Na^+ ion.[2,3,36] Recent studies suggest a similar binding order for Na^+ and Asp in Glt_{Ph}.[9,12,29] We propose that at low Na^+ concentrations, Arg397 is positioned so it is unable to interact with the β-carboxyl group of Asp while Na^+ binding causes a conformational change in the substrate binding site, resulting in a repositioning of the Arg side chain for optimal coordination of the substrate (Figure 5C).

In conclusion, we have developed a semisynthesis of Glt_{Ph}. The semisynthesis allows the precise engineering of Glt_{Ph} through the use of chemical synthesis for the incorporation of unnatural amino acids and modifications of the peptide backbone. We anticipate that these unnatural modifications introduced using semisynthesis will be extremely useful in investigating functional mechanisms in Glt_{Ph}. Recent years have seen a rapid increase in the available amount of structural information about transporters. We anticipate that the semisynthetic approaches that we developed for Glt_{Ph} will be applicable to these membrane transport proteins and will permit a detailed analysis of the transport mechanisms in these transporters.

■ ASSOCIATED CONTENT

Supporting Information

A figure showing the RP-HPLC and ES-MS data for the synthetic peptides used for the semisynthesis of WT and R397 Cit Glt_{Ph}. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

P.J.F. and F.I.V. designed the experiments. P.J.F. and A.W.A. performed the experiments. P.J.F. and F.I.V. analyzed the data and wrote the manuscript.

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Notes

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■ ABBREVIATIONS

TM, transmembrane helix; HP, hairpin loop; SPPS, solid phase peptide synthesis; NCL, native chemical ligation; DDM, dodecyl β-D-maltopyranoside; Tx-100, Triton X-100; NLS, *N*-lauryl sarcosine; MESNA, 2-mercaptoethanesulfonic acid; PAM, phenylacetamidomethyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HF, hydrofluoric acid; Cit, citrulline; Fos-12, dodecylphosphocholine; GST, glutathione *S*-transferase; SEC, size exclusion chromatography; TBOA, DL-threo-β-benzyloxyaspartic acid; PDB, Protein Data Bank.

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