Asp$^{22}$ drives the protonation state of the *Staphylococcus epidermidis* glucose/H$^+$ symporter

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The *Staphylococcus epidermidis* glucose/H$^+$ symporter (GlcP$_{Se}$) is a membrane transporter highly specific for glucose and a homolog of the human glucose transporters (GLUT, SLC2 family). Most GLUTs and their bacterial counterparts differ in the transport mechanism, adopting unipolar and sugar/H$^+$ symport, respectively. Unlike other bacterial GLUT homologs (for example, XylE), GlcP$_{Se}$ has a loose H$^+$/sugar coupling. Asp$^{22}$ is part of the proton-binding site of GlcP$_{Se}$ and crucial for the glucose/H$^+$ co-transport mechanism. To determine how pH variations affect the proton site and the transporter, we performed surface-enhanced IR absorption spectroscopy on the immobilized GlcP$_{Se}$. We found that Asp$^{22}$ has a $pK_a$ of 8.5 ± 0.1, a value consistent with that determined previously for glucose transport, confirming the central role of this residue for the transport mechanism of GlcP$_{Se}$. A neutral replacement of the negatively charged Asp$^{22}$ led to positive charge displacements over the entire pH range, suggesting that the polarity change of the WT reflects the protonation state of Asp$^{22}$. We expected that the substitution of the residue Ile$^{105}$ for a serine, located within hydrogen-bonding distance to Asp$^{22}$, would change the microenvironment, but the $pK_a$ of Asp$^{22}$ corresponded to that of the WT. A167E mutation, selected in analogy to the XyIE, introduced an additional protonatable site and perturbed the protonation state of Asp$^{22}$, with the latter now exhibiting a $pK_a$ of 6.4. These studies confirm that Asp$^{22}$ is the proton-binding residue in GlcP$_{Se}$ and show that charged residues in its vicinity affect the $pK_a$ of glucose/H$^+$ symport.

The cellular uptake of monosaccharides, polyols, and other small carbon compounds across the membranes of eukaryotic cells is an essential physiological process. In humans, their transport is facilitated by specialized transporters that are members of the GLUT family (SLC2 gene family), which belongs to the major facilitator superfamily (MFS), one of the largest protein families with over 10,000 members (1). There are 14 human GLUT proteins; they share sequence identity with 19–65% identity but differ in tissue distribution, substrate selectivity, and substrate affinity to meet local physiological needs. GLUTs have been implicated in several diseases, including cancer (2, 3) and diabetes (4). In plants, monosaccharide and sucrose transporters play a fundamental role in stress responses and developmental processes, including seed germination and balanced growth (5, 6). Yeast glucose transporters are essential for glucose uptake and metabolism during sugar fermentation and alcohol production (7). Most GLUT proteins catalyze the facilitative (energy-independent) bidirectional transfer of their substrates across membranes, and they may exhibit either symmetric or asymmetric transport kinetics. On the other hand, most bacterial GLUT homologs, such as GlcP$_{Se}$ (8), AraE (9), GalP (10), and XylE (11), are H$^+$ symporters and depend on the electrochemical proton gradient ($\Delta \mu H^+$).

GLUTs and their homologs have 12 transmembrane $\alpha$-helic residues organized as two domains (N- and C-halves), which form a central, solvent-accessible, cavity: the substrate-binding site. MFS proteins presumably share an alternating access mechanism of transport in which the substrate-binding site alternately opens to either side of the membrane (12). The available X-ray crystal structures of GLUTs and their homologs are consistent with this model; they include structures for the inward-facing (open to the cytoplasmic side) (13–15), outward-facing (open to the periplasmic side) (11, 16), and occluded conformations (17–19).

The crystal structure of the *Staphylococcus epidermidis* glucose/H$^+$ symporter, a bacterial GLUT homolog, was solved in the inward-facing conformation (8). GlcP$_{Se}$ shares high sequence identity (27–34%) and homology (49–58%) with the human GLUTs, is highly specific for glucose, and is inhibited by the well-characterized inhibitors of human GLUTs phloretin, cytochalasin B, and forskolin (8). Residues with potential H$^+$-binding capabilities were identified; however, unlike some bacterial MFS transporters, for example, XyIE, GlcP$_{Se}$ has only two charged residues in the H$^+$-binding site: Asp$^{22}$ in helix 1, involved in the proton-binding site, and Arg$^{102}$ from helix 4, which may form a salt bridge with Asp$^{22}$ when the proton is absent (Fig. 1). Indeed, crystal structures of XyIE obtained at acidic (pH 5.8) and alkaline (pH 9.6) pH were in the inward- and outward-facing conformations, respectively, with Asp$^{27}$ (the equivalent of Asp$^{22}$ in GlcP$_{Se}$) interacting with Arg$^{133}$ (the equivalent of Arg$^{102}$ in GlcP$_{Se}$) in the outward-facing conformation but not in the inward-facing conformation (11, 20).

This article contains supporting information.

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For both GlcPSe and XylE, a transport mechanism is discussed where the transition between the inward-facing and outward-facing conformations involves a 24° relative rotation of the N- and C-domains around an axis that passes through the substrate-binding site, suggesting that translocation of glucose is coupled with conformational changes associated with the transition between the outward- and inward-facing conformations. The involvement of Asp22 in the H⁺-binding site was shown by the loss of a pH effect on entrance counterflow in proteoliposomes and of active glucose transport in right-side-out vesicles (8). It was proposed that, in the absence of a proton, Asp22 and Arg102 form a salt bridge to juxtapose helices 1 and 4, thereby opening the substrate cavity wide. When the proton binds to Asp22, the salt bridge breaks, and helices 1 and 4 rearrange to decrease the size of the substrate cavity; this then lowers the energetic barrier of the transporter’s conformations, and the glucose is transported (8). This proposal has been supported by structural studies and molecular dynamics simulations of XylE (11, 20). Bazzone et al. (21) suggested that H⁺/sugar coupling is incomplete in GlcPSe, leading to the unexpected finding that, depending on the experimental conditions, GlcPSe can function as a symporter or show partially uncoupled transport modes. It has been proposed that the existence of an H⁺-binding site is necessary but not a sufficient requirement for H⁺/sugar symport (21), leading to the hypothesis that an additional structural element for strictly coupled transport would be necessary, such as an acidic residue.

Here we study the pH- and substrate-dependent conformational changes in purified GlcPSe by IR spectroscopy for WT and several H⁺-binding site mutants, including A167E. This mutation is at a position proposed to play the role of the additional structure element crucial for strict symport. The suggestion is based on the analogy with the residue at position 206 in XylE (22). The pKa of Asp22 was determined by monitoring pH-induced changes of the COOH vibrational modes of the purified GlcPSe immobilized on a gold layer in an attenuated total reflectance (ATR) cell in analogy to the surface-enhanced absorption IR spectroscopy (SEIRAS) approach used to study the pKa value of Glu325 in LacY, the most-studied MFS transporter (23). The effect of the mutations on the pKa value of Asp22 is discussed.

**Results**

**SEIRAS spectra and assignment of the signal for residue Asp²²**

IR difference spectra have been measured to monitor the protonation/deprotonation reactions and coupled conformational changes. Fig. 2 shows the difference spectra that were obtained by subtracting spectra acquired from samples equilibrated at pH 5.5 from spectra collected of samples equilibrated at a pH that completely or almost completely deprotonates Asp²² (pH 9.5 or 8.5, respectively), in the absence of glucose. The grey line shows the inverse reaction and thus a high degree of reaction reversibility.

The difference spectra reflect structural reorganization within GlcPSe caused by the shift in pH, including changes in the protein backbone conformations and the protonation state of individual side chains; for example, the COOH vibrational mode of protonated acidic residues is typically ~1750 cm⁻¹ (24, 25). Distinguishable positive and negative signals reflect these conformational changes. The position of the COOH vibrational mode that is specific for the hydrophobicity of the environment of a protonated acidic residue is only 4 cm⁻¹ higher than the one recently observed for Glu³² in LacY (23, 26), indicating a similar microenvironment (Fig. 2A). The difference spectra for the same pH step of D22A GlcPSe (Fig. 2B) is almost identical to the WT one, except for the important signal at 1750 cm⁻¹, confirming that this signal arises from Asp²², a residue previously suggested to play a similar role to Glu³² in LacY (23, 26).

The spectra of both WT and D22A GlcPSe have signals in the so-called amide I region, between 1690 and 1620 cm⁻¹, representing contributions of the protein backbone and individual side chains. The position of the amide I backbone signal is specific for the type of secondary structure. Signals at ~1650 cm⁻¹ correspond to α-helices, and those at ~1642 cm⁻¹ indicate...
In an analogous study performed on LacY with the same approach (23), the presence of 4-nitrophenyl-\(R\)-d-galactopyranoside did not influence the conformational changes. However, it is noted that 4-nitrophenyl-\(R\)-d-galactopyranoside shows very different transport rates, and direct comparison is difficult.

I105S was previously studied in GlcPse, to investigate why human GLUT2, which seemingly can have a proton-binding site (Asp\(^{22}\) of GlcPse is Asp\(^{27}\) of GLUT2), is a unipporter and not a proton symporter (8). Close to the proton-binding site, GlcPse has Ile\(^{161}\), whereas in the same position, GLUT2 has Ser\(^{161}\), which can come within hydrogen bond distance from Asp\(^{27}\). Therefore, it was proposed that I105S GlcPse would exhibit impaired active glucose transport. Indeed, this mutant had no active glucose transport in right-side-out vesicles and had an attenuated pH effect in the entrance counterflow glucose transport in proteoliposomes, although much less drastically than the D22N mutant (8). Nonetheless, we find that I105S substitution does not perturb the signal at 1750 \(\text{cm}^{-1}\), although some changes in the amide I and II region suggest smaller structural changes (Fig. 3C).

Interestingly, for A167E GlcPse (Fig. 3D), the signal at 1750 \(\text{cm}^{-1}\) and the signal at 1724 \(\text{cm}^{-1}\) are lost in the absence of glucose and restored at 2 \(\text{cm}^{-1}\) lower position in the presence of the substrate. The introduced Glu\(^{167}\) changes the environment of Asp\(^{22}\), pointing toward an interaction between Asp\(^{22}\) and Glu\(^{167}\), eventually introduced by a conformational change. In XyIE, Asp\(^{27}\) (the equivalent of Asp\(^{22}\)), interacts with Glu\(^{206}\) (the equivalent of Ala\(^{167}\) in GlcPse), at both acidic (pH 5.8) and alkaline (pH 9.6) pH, although E206A has only a slight reduction in transport activity (11, 20). Residue 167, identified in analogy to XyIE\(_{E. coli}\), was suggested to play a critical role in the pK switch of the H\(^+\)--binding site to acidic values, to facilitate H\(^+\) release (21, 22). Nevertheless, the environments of the proton-binding sites in XyIE and GlcPse vary significantly, with that of XyIE being mostly polar (amino acid residues Ser\(^{159}\), Ser\(^{203}\), Arg\(^{130}\), and Glu\(^{206}\)), including structural water molecules, whereas that in GlcPse is mostly hydrophobic, the only charged residue being the critical Arg\(^{102}\). Introducing A167E mutation in GlcPse seems very disruptive, especially given the proximity of the hydrophobic bulky residue Ile\(^{105}\). Therefore, in analogy to XyIE proton-binding site, we also generated the double mutant A167E/I105G; structural modeling suggested a better accommodation of A167E substitution in the double mutant than in the single mutant A167E (Fig. S2).

Investigation of the double mutant A167E/I105G (Fig. 3F) pointed to major structural changes. The negative signal at 1750 \(\text{cm}^{-1}\) was lost in all cases and replaced by a positive feature at 1732–1720 \(\text{cm}^{-1}\). Because positive signals in the difference spectra corroborate with the data collected at more alkaline pH values, there is no easy explanation for this new signal that is in the spectral region typical for protonated acidic residues. It indicates the presence of a protonated residue at alkaline pH, in an environment whose pH-induced changes lead to deprotonation. This would point to a strong reorganization of the residue’s microenvironment, enabling a stabilization of the protonated residue despite the buffer alkaliatization. The introduced Glu\(^{167}\) residue could be contributing here, and also
Asp^{22} could be showing a different signature caused by the modified environment in the mutant.

\[ pK_a \] of Asp^{22} in GlcP_{Se}

The \( pK_a \) of Asp^{22} in WT and different mutants, in the presence and absence of glucose, was determined by plotting the difference in signal intensity at 1750 cm\(^{-1} \) versus pH (Fig. 4). From the ATR fit observed (red and black, with and without sugar, respectively), Asp^{22} has a \( pK_a \) of 8.5 ± 0.1, a value that agrees remarkably well with the \( pK_a \) obtained previously by Bazzone et al. (21) for the glucose transport, through a different approach.

The mutant I105S GlcP_{Se}, which has a serine in the proximity of the H\(^+\)-binding site, would have been a possible candidate to perturb the proton binding. However, the \( pK_a \) of this mutant (8.5 ± 0.1) is similar to that of WT GlcP_{Se}, showing that Asp^{22} is not interacting with Ser^{105}. The comparison of the data with and without glucose reveals that the \( pK_a \) of Asp^{22} in WT and I105S is independent of glucose.

For the A167E mutant, the scenario is more complex. Whereas Asp^{22} has a \( pK_a \) of 8.5 in the presence of glucose, there is no signal from Asp^{22} in the absence of sugar, pointing to a major structural reorganization in the environment of the residue, which might be locked in a certain conformation because of the steric disruptions in the proton-binding site (Fig. S2).

In the A167E/I105G mutant, the signal of the acidic residue at 1750 cm\(^{-1} \) is lost irrespective of the glucose presence. Most likely Asp^{22} is deprotonated even at pH 5.5, the lowest pH applied in our experiments.

**Isothermal titration calorimetry (ITC) of WT and proton-binding site mutants**

We examined the glucose binding affinity to purified GlcP_{Se} WT and proton-binding site mutants, at pH 7.5 and 9.5, using isothermal calorimetry (ITC). Previous studies showed a lower \( K_d \) of glucose in GlcP_{Se} at alkaline pH compared with that at acidic pH (21). We found a similar trend by ITC, with \( K_d \) of glucose at pH 7.5 being ~7-fold higher than that at pH 9.5 for the
WT transporter (Fig. S3 and Table S1). Nevertheless, glucose dissociation constants measured by ITC (Kd 11.7 ± 1.7 μM at pH 7.5 and 1.75 ± 0.93 μM at pH 9.5; Table S1) were ~1000-fold lower than those obtained in solid support membrane (SSM) experiments (Kd of 12.3 mM at pH 5.5 and 2.6 mM at pH 10) (22) and comparable with the Km of glucose previously reported (~30 μM) (8). Interestingly, mutations in the proton-binding site significantly reduced the difference in glucose affinity between pH 7.5 and 9.5, reversing the trend found with the WT. Thus, D22A, D22N, and A167E GlcPSe had 1.4–2-fold higher Kd at pH 9.5 than at pH 7.5 (Table S1). Intriguingly, compared with WT, all of these proton-binding site mutants had better glucose affinity at pH 7.5, whereas the reverse was true at pH 9.5 (Table S1).

**SSM-based electrophysiology**

The pH dependence of the transient currents generated by WT GlcPSe after a sugar concentration jump has previously shown an atypical behavior: a transient current of positive polarity at acidic pH and negative polarity at alkaline pH (21). The GlcPSe mutant A167E/I105G shows a very similar behavior, with the mutant A167E/I105G at alkaline pH. The transient currents with both positive and negative amplitudes are not affected by the lipid-to-protein ratio (LPR) (Fig. S4), indicating a characteristic of pre–steady-state reactions. Unlike in experiments with the WT GlcPSe, in the mutant A167E/I105G, the positive phase overcompensates the amplitude with the negative polarity also at alkaline pH.

Consequently, the normalized total charge does not change the polarity (21).

One feature that distinguishes the GlcPSe from the more classical *Escherichia coli* sugar transporters, e.g. LacY (30, 31), XylE, and FucP, is that the affinity toward sugar decreases with higher H⁺ concentration (lower pH). For the WT GlcPSe, a Kd of 2.6 mM at an alkaline pH of 10 is reported and 12.3 mM at an acidic pH of 5.5 (21). The mutant A167E/I105G shows a comparable Kd of 11.4 ± 3.3 mM at an alkaline pH of 8.5 (Fig. 5C).

**Glucose transport activity of GlcPSe**

WT, D22A, A167E, and I105S/A167E GlcPSe, were expressed in JM-1100 *E. coli* cells, which lack original glucose transporters (32), and the transport activity was measured as glucose uptake in the whole cells. We found that D22A was a dead mutant, whereas A167E and I105S/A167E mutants retained 13.6 and 34.5% of WT activity, respectively (Fig. S5).

**Discussion**

The molecular basis of the coupling between the proton and substrate transport in MFS transporters has been the object of intense research (20, 21, 33, 34), with LacY featuring prominently as a model. In LacY, the two residues critical for proton symport are Glu325 and Arg102, located in TM helices 10 and 9, respectively, (13, 23, 35), and the proton and substrate sites are adjacent, providing a structural basis for the proton–substrate coupling. Arg102 of LacY is important for deprotonation of Glu325 (24, 36). In GlcPSe and related bacterial sugar porters, like XylE, AraE, and GalP, the proton-binding residue (Asp22 in GlcPSe) is located in TM helix 1, and the substrate and proton sites are separated (Fig. S6), making the proton–substrate coupling more obscure (8, 11).

SEIRAS investigations of the proton site in GlcPSe revealed that the pKa of Asp22 is 8.5, confirming previous studies (21). Unlike XylE, whose proton-binding site (Asp27) is surrounded by polar residues (Ser98, Ser102, Arg133, and Glu206) and even water molecules (11, 20), the only polar residue near Asp22 is Arg102 (corresponding to Arg133 in XylE), which presumably plays the role of Arg302 in LacY (8, 20). Buried acidic or basic residues can have markedly different pKa compared with when they are solvent-accessible (36). For instance, the pKa of E325 in LacY was 10.5 (27), or that of Glutamates at various internal positions in a staphylococcal nuclease ranged from 5.2 to 9.4 (37). Buried Lys residues can have a pKa as low as 5.3 (38), raising the possibility that Arg102 may have a lower pKa, as required by its proposed role in deprotonating Asp22.

When discussing the effect of pH on a proton transporter, there are two considerations. First, the pH may stabilize particular transporter conformations by affecting polar interactions and salt bridges. Second, the proton binding or release can favor or induce conformational changes in the transporter. SEIRAS studies here capture the pH-induced conformational changes independent of proton binding. Thus, D22A GlcPSe lacks the proton-binding site, but variations in pH still induce conformational changes in the transporter, which get significantly abated in the presence of glucose (Fig. 3B, spectra a–c). ITC shows an attenuation of the pH effect on glucose affinity in...
D22A versus the WT transporter, although it has the opposite impact on glucose affinity (Table S1). D22A abrogates $\Delta \mu H^+$-dependent transport, and its glucose transport activity is abolished (Fig. S4A). Nevertheless, it binds glucose better than the WT transporter at pH 7.5 (Table S1), implying that glucose binding alone is insufficient to trigger the conformational change necessary for transport. The increase in glucose affinity with pH lead to the proposal that the proton and substrate are loosely coupled in GlcPSe and that an additional element, like Glu206 in XylE, may be required for tight symport (21).

Like D22A, D22N and A167E also display reversed glucose affinities at pH 7.5 and 9.5, compared with the WT. Furthermore, all these mutants have 3- or 5-fold higher glucose affinity than the WT at pH 7.5, whereas glucose uptake in whole cells is abolished (D22A) or 86% decreased (A167E) (Fig. S5). Thus, the increase in glucose affinity does not translate in higher transport activity, even when the proton site is present, as in A167E. Nevertheless, structural modeling predicts that substitution of Ala167 with the charged long side chain of Glu disrupts the proton-binding site significantly, given the neighboring Ile105 (Fig. S2). This perturbation is reduced in the double mutant A167E/I105G, where the bulky hydrophobic side chain is removed.

The IR spectra of A167E mutant in the absence of glucose do not change significantly with pH (Fig. 3D, spectra a–c), possibly indicating that the transporter is blocked in a particular conformation. Glucose restores pH-dependent conformational variability, as evidenced by IR data (Fig. 3D, spectra d–f) and the ITC results (Fig. S3 and Table S1). On the other hand, A167E/I105G mutant responds to pH changes, whether glucose is present or not, suggesting that the double mutant is better behaved than the A167E mutant. Indeed, the transport assay showed that A167E/I105G mutant had better glucose transport activity than A167E (Fig. S5A). Expectedly, the $K_a$ of transport for A167E/I105G of 6.8 (Fig. 5) is almost 2 pH units lower than that of the WT (Fig. 4), suggesting higher solvent exposure of the proton-binding site in this mutant.

LacY, FucP, and XylE, all show the decrease of substrate affinity with pH displayed by A167E mutant (Table S1) (22), presumably a hallmark of tightly coupled symporter. However, the same relationship between pH and glucose affinity is also present in D22A, lacking the proton-binding residue. Thus, even before proton binding, the proton gradient may play a role in selecting transporter conformations with particular affinities for the substrate.

Interestingly, similarly to GlcPSe, AraE, a pentose/proton symporter (9), and GalP, a hexose-proton symporter (10), both bacterial GLUT homologs, lack Glu206 of XylE (Ala167 in GlcPSe) having instead Leu184 or Ile177, respectively. The proton-binding sites of AraE and GalP resemble that of GlcPSe, although they do have the equivalent of Ser102 of XylE, Ser106 (AraE), or Ser99 (GalP) (Fig. S6). Given the overall proton environment, AraE and GalP may also exhibit high $K_a$ for their carbohydrate transport. Whether they behave like GlcPSe (increased sugar affinity with increased pH) or like XylE and LacY (decreased sugar affinity with higher pH) remains to be established.

In humans, GLUT2 has D47 as the corresponding residue for Asp22 of GlcPSe, and the only significant change in the proton-binding site is Ser161 instead of Ile105. Nevertheless, I105S GlcPSe data (Fig. 3C) and a previous report (21) indicated no changes in the $K_a$ of transport compared with that of WT GlcPSe. This raises the possibility that GLUT2 may be capable of proton symport in certain conditions, as shown for GLUT12, which has glutamate in the position of Asp22 of GlcPSe.

Conclusion

The presence of a residue with a high $K_a$ value can be crucial to transport proteins (39, 40). The high $K_a$ value is based on the hydrophobicity of the microenvironment and will be manipulated because of conformational changes during transport. The presence of such residues with a high $K_a$ was observed before in other membrane protein, for example Rhodopsins (21) and cytochrome c oxidase (41). Here Asp22 seems to play this role with a $K_a$ value of 8.5. Interestingly this $K_a$ can be corroborated with that of glucose transport. Residues within the hydrogen bonding distance to the proton site actively interact with Asp22 and change the $K_a$ of the transport as well as the affinity of glucose and its pH dependence. These residues including an arginine, often found as a partner of acidic residues when the $K_a$ is upshifted (41). SEIRAS of MFS proton symporters can monitor directly the protonation/deprotonation of the transporters and the backbone conformational changes associated with substrate and proton binding, providing invaluable insight into the MFS transport mechanism.
Experiment procedures

Protein purification

Site-directed mutagenesis GlcPSe was performed on the pBAD plasmid constructs of WT with C-terminal hexahistidine-tagged proteins and verified by DNA sequencing (42). WT, D22A, D22N, I105S, and A167E GlcPSe were expressed in C41 E. coli cells and purified as previously described (8).

Glucose transport assay

Glucose transport assay of WT and mutant GlcPSe in whole cells was performed as previously described (8). GlcPSe WT and D22A, A167E, and I105S/A167E mutants in pBAD vector and empty pBAD vector were transferred into E. coli JM-1100 cells. The cells were grown in 10 ml of LB medium with 50 μg/ml ampicillin at 37°C with shaking (200 rpm). At cell A600 nm of 0.6–0.8, GlcPSe expression was induced with 0.3 mM l-arabinose, and cells were grown for 2–3 h. The cells were collected by centrifugation at 4000 × g, 5 min, and the cell pellet was resuspended in 5–10 ml of PBS buffer (10 mM Na2HPO4, 1.8 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) to wash the cells. This was followed by another centrifugation and resuspension of the cell pellet to A600 nm ~3.0 in 0.1 mM KP, pH 7.5, 10 mM MgSO4. Each assay had 50 μl of this cell solution. The transport was initiated by the addition of 50 μM [14C]glucose and stopped by the addition of 3-ml ice-chilled Quench buffer (0.1 mM KP, 0.1 mM LiCl, pH 5.5), followed by filtration on cellulose nitrate membrane filters (catalog no. 7184-002; Whatman plc), and two more washes with 3 ml of quench buffer. The filters were then transferred into scintillation vials, combined with 10-ml scintillation mixture (BioSafeII; Research Products International, Mount Prospect, IL, USA), and after brief vortexing, the radioactivity was measured with a scintillation counter (Tri-carb 2900TR; PerkinElmer). The cells expressing the pBAD vector without GlcPSe provided the control for the background glucose transport activity. To quantify protein expression in JM-1100 cells, cell pellets from 3 ml of cell solution A600 nm 3.0 (same as that used for transport assay) were resuspended in 300 μl of PBS buffer, combined with 300 μl of 2X SDS-PAGE sample loading buffer, and disrupted by sonication. For Western blotting, 10 μl of the broken cell solution were loaded in each SDS-PAGE gel well. We used horseradish peroxidase–conjugated pentahistidine antibody (Qiagen) and visualized the protein bands by chemiluminescent substrate (Thermo Scientific), according to the manufacturer’s instructions.

ITC of GlcPSe

The experiment was performed on Affinity ITC (TA Instruments, New Castle, DE, USA). The sample cell contained 300 μl of purified protein at 7.5 mg/ml (0.15 mM) in 0.1 M KP buffer at pH 7.5 or 9.5, with 0.1 M NaCl, and 0.05% (w/v) dodecylmaltoside (DDM). Glucose, at 1.5 mM concentration, dissolved in the corresponding buffer, was titrated as 3 μl/injection in 30 injection steps. The data were processed with NanoAnalyze (TA Instruments).

Surface modification of the silicon crystal and protein immobilization

Before the formation of the gold film, the crystal was polished with finer grade 0.3-μm alumina, follow by rinsing with copious amounts of Millipore water, acetone, and water again. On the surface of a silicon ATR crystal, a thin gold layer was formed by chemical deposition as described previously (43). The crystal was dried under an argon stream, and 40% NH4F (w/v) was added for 1 min to remove the silicon oxide layer and to terminate with hydrogen; finally the surface was rinsed and dried again. The crystal was heated at 65°C for 10 min together with the plating solution. The composition of the solution was a 1:1:1 mix (v/v/v) of 15 mM NaAuCl4, 150 mM Na2SO3, 50 mM Na2S2O3, 50 mM NH4Cl, and 2% HF (w/v: 1 ml). After reaching the temperature, the prism was covered with the solution for 40 s and stopped by washing the plating solution with water, followed by drying with a stream of argon.

After formation of gold layer on the silicon crystal, a nickel–nitritoltriacetic acid self-assembled monolayer was adapted from Refs. 43 and 44. First, 1 mg/ml of 3,3-dithiodipropionic acid di(N-hydroxysuccinimide ester) in DMSO was allowed to self-assemble for 1 h. After monolayer formation, excess 3,3-dithiodipropionic acid di(N-hydroxysuccinimide ester) was washed away with DMSO, and the crystal was dried under an argon stream. Afterward, the self-assembled monolayer was immersed with 100 mM N,N,N′,N′-bis(carboxymethyl)-l-lysine in 0.5 M K2CO3 at pH 9.8 for 3 h and then rinsed with water. Finally, the surface was incubated in 50 mM Ni(ClO4)2 for 1 h. After washing with water, 2–35 mg/ml of the protein GlcPSe dissolved in 50 mM phosphate buffer containing 0.05% (w/v) n-dodecyl-β-maltoside was deposited on the modified gold surface for 1 h.

IR spectroscopy

A simultaneous acquisition of FTIR spectra in the ATR mode with perfusion of solutions was used. A silicon crystal with 3-mm surface diameter was used as a single reflection ATR unit. The experiments were carried out with a Bruker Vertex 70 FTIR spectrometer (Globar source, KBr Beamsplitter, mercury cadmium telluride detector) at 8-mm aperture and 40-kHz scanner velocity. The measurements were carried out at ~7°C, and the solutions were kept on ice before use. Before each perfusion step, the input tube was carefully washed with water and buffer, and the pump speed was kept constant at a flow rate of 0.2 ml/min.

Secondary structure determination and protein orientation on the surface

SEIRAS spectra have been obtained for the protein immobilized via its His tag on a silicon crystal, covered with a gold nanostructure. The immobilization ensures that the protein concentration remains stable during the experiments. The deconvolution of the amide I band of the absorbed protein (Fig. S7) confirmed the structural integrity of the protein after immobilization. For each experiment the immobilization was followed spectroscopically to ensure a high degree of reproducibility.
The orientation of the amide I mode was studied with polarized IR light, showing that at pH 7.5 the protein is oriented perpendicular to the ATR crystal, and at pH values higher and lower the protein shows some inclination, most likely because of the charges on the surface of the protein (Table S2). Nevertheless, the secondary structures of each pH value and of the mutants were found to be unchanged within the experimental error of the technique (3–5%) (Fig. S6).

**Difference spectra of GlcPSe in the presence or absence of glucose**

To monitor pH-induced difference spectra, we used one perfusion buffer with constant pH value 5.5 (50 mM KP, 200 mM NaCl, 0.05% (w/v) DDM) and a second perfusion solution with the same composition but at different pH values ranging from 7.5 to 10.5. First, the system was equilibrated with the KP (pH 5.5) for 20 min, then a spectrum was recorded as background, and the perfusion solution was changed to the second solution (pH 7.5–10.5). After 20 min (pH 7.5–10.5) minus pH 5.5 difference spectra were recorded. The new state of the protein was recorded as background, and the solution was changed to pH 5.5. Again, after 20 min, the pH 5.5 minus (pH 7.5–10.5) difference spectra were obtained. The same experiments that have been performed in the absence of glucose were performed in the presence of glucose. Glucose was dissolved in 50 mM KP, 200 mM NaCl, 0.05% (w/v) DDM (pH 5.5) at a final concentration of 100 μM. The data were normalized based on the absorbance spectra obtained at the beginning of each experiment, when the data from different samples had to be compared.

**Reconstitution of GlcPSe into proteoliposomes**

Reconstitution of purified proteins (2 mg/ml) was carried out with *E. coli* phospholipids (*E. coli* polar lipid extract; Avanti Polar Lipids, Alabaster, AL, USA). Preformed liposomes (0.2–2 ml, 10 mg/ml) dissolved in 1% (w/v) octyl-glucoside and the protein suspension were mixed on ice to their respective concentration, to result in different LPR. The LPR 5 sample was used for all SSM measurements of the pH and glucose titrations.

All proteins were reconstituted using overnight incubation in 400 mg/ml BioBeads (SM-2 adsorbent media; Bio-Rad) at 4°C. After reconstitution, the samples were diluted to 2.5 mg/ml lipid concentration, frozen in liquid nitrogen, and stored at −80°C.

**SSM-based electrophysiology**

SSM measurements were performed as described previously (21, 30, 45, 46). The nonactivating buffer and activating solutions were prepared in 100 mM KP, 1 mM DTT at pH 7.5. The nonactivating solution contained 30 mM xylose, the activating solution contained 30 mM glucose for the pH jumps, and the pH of 8.5 was applied for the glucose titration. Glucose concentration and pH jumps experiments were carried out respectively on the same sensor.

**Data availability**

All data shown are available in the article and the supporting information.

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**Abbreviations**—The abbreviations used are: GlcPSE, glucose permease from *S. epidermidis*; SEIRAS, surface-enhanced absorption infrared spectroscopy; ATR, attenuated total reflectance; MFS, major facilitator superfamily; SSM, solid support membrane; H/D, hydrogen/deuterium; ITC, isothermal calorimetry; DDM, dodecylmaltoside; LPR, lipid-to-protein ratio.

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