Arg302 governs the $pK_a$ of Glu325 in LacY

Natalia Grytsyka, Ana Filipa Santos Seiça, Junichi Sugihara, H. Ronald Kaback, and Petra Hellwig

Lactose permease is a paradigm for the major facilitator superfamily, the largest family of ion-coupled membrane transport proteins known at present. LacY carries out the coupled stoichiometric symptom of a galactoside with an H$^+$, using the free energy released from downhill translocation of H$^+$ to drive accumulation of galactosides against a concentration gradient. In neutrophilic Escherichia coli, internal pH is kept at ~7.6 over the physiological range, but the apparent $pK$ ($pK_{app}$) for galactoside binding is 10.5. Surface-enhanced infrared absorption spectroscopy (SEIRAS) demonstrates that the high $pK_a$ is due to Glu325 (helix X), which must be protonated for LacY to bind galactoside effectively. Deprotonation is also obligatory for turnover. Here, we utilize SEIRAS to study the effect of mutating residues in the immediate vicinity of Glu325 on its $pK_a$. The results are consistent with the idea that Arg302 (helix IX) is important for deprotonation of Glu325.

Glu325 plays a uniquely important role in the mechanism of coupling between galactoside and H$^+$ translocation during syndport by the lactose permease of Escherichia coli (LacY). Replacement with various neutral side chains leads to a syndporter that is completely defective in all reactions involving coupled H$^+$ translocation, with no effect on transmembrane galactoside exchange (1, 2) or binding (3, 4). Notably, the alternating access component of the mechanism, which is represented by transmembrane exchange of galactosides, is driven by sugar binding and dissociation, and not by the H$^+$ electrochemical gradient $\Delta\mu_{H^+}$ (reviewed in ref. 5). Furthermore, galactoside binding exhibits an apparent $pK$ ($pK_{app}$) of 10.5, which is abolished in mutant E325A, where high-affinity binding is observed up to pH 11, where LacY begins to destabilize (4). Thus, the galactoside-binding site is remarkably stable to alkaline pH, but Glu325 must be neutralized to elicit this property. Of paramount importance, it has also been demonstrated directly by surface-enhanced infrared absorption spectroscopy (SEIRAS) that the $pK_a$ of Glu325 itself is 10.5, the same as that obtained for galactoside binding, and that this perturbed $pK_a$ is due to a local hydrophobic environment (6). Thus, a main, unanticipated feature of coupling is that galactoside binding is dependent specifically upon protonation of Glu325, and protonation acts to neutralize an inhibitory negative charge on this side chain.

Of course, LacY must also deprotonate for turnover to occur. However, transmembrane exchange reactions, which represent alternating access, do not involve $\Delta\mu_{H^+}$ and exhibit pH profiles similar to that observed for galactoside binding (7, 8). Thus, during exchange, LacY remains protonated (5). In neutrophilic E. coli, internal pH is kept at ~7.6 over the physiological range (9, 10) and the $pK_{app}$ for galactoside binding is 10.5 (6, 11, 12). Therefore, to deprotonate only ~50% of LacY, the $pK_a$ would have to decrease by three orders of magnitude, which would be very inefficient. The alternative of decreasing cytosolic H$^+$ concentration seems even more unlikely.

So how can deprotonation of LacY occur at physiological pH with a $pK_a$ of 10.5? One possibility is a structural change that exposes protonated Glu325 to a more aqueous local environment. Another is to bring Arg302 (helix IX) close to Glu325 (helix X). Although the two residues are 6–7 Å apart with the hydroxyl group of Tyr236 between them in the current structure, the double-Cys mutant R302C/E325C exhibits pyrene excimer fluorescence (13) and double-His R302H/E325H binds Mn(II) with micromolar affinity (14). Thus, Arg302 and Glu325 may be in closer proximity in another conformation of LacY. In this regard, like the Glu325 neutral mutants (2), neither R302S nor R302A LacY performs active transport, but both mutants bind galactoside and catalyze transmembrane exchange. For these reasons, it was suggested that positively charged Arg302 may be important with respect to deprotonation of Glu325 (15), and further evidence supporting this possibility has been presented (16).

Although reaction-induced SEIRAS may be useful for determining the $pK_a$s of acidic side chains (6, 17), other functional groups in proteins are often obscured by contributions from protein backbone reorganization caused by the induced reaction (18). In view of the critical importance of Glu325 and the possibility that Arg302 may be important for deprotonation, we have tested the effect of mutating Arg302 and other side chains in the immediate vicinity of Glu325 on its $pK_a$ based on the notion that side chains that interact chemically with Glu325 and/or change the local environment should alter the $pK_a$ (Figs. 1 and 2). The results provide further evidence that Arg302 may interact with Glu325 to drive deprotonation.

Results

SEIRAS Spectra. The spectra presented in Fig. 3A are difference spectra obtained by subtracting data obtained from samples equilibrated at pH 7.0 from samples obtained at a pH that...
completely or almost completely deprotonates Glu325 (pH 10.9). The difference spectra reflect all reorganization within LacY due to the shift in pH, including conformational changes in the backbone and changes in the protonation state of individual side chains, for example, the COOH vibrational mode of Glu325 assigned to 1,746 cm$^{-1}$ (6). Positive and negative signals can be distinguished reflecting these conformational changes. The parts of the protein that do not change with pH are omitted from the difference spectra (17).

Data interpretation is possible because of the availability of a large number of infrared studies on proteins and model systems. The spectra are characterized generally by signals in the so-called “amide I” region between 1,690 and 1,620 cm$^{-1}$ that involve backbone contributions, as well as contributions from individual side chains. The position of the amide I backbone signal is specific for the type of secondary structure, and signals at $\sim1,650$ cm$^{-1}$ correspond to $\alpha$-helices. These contributions are partially obscured by disordered structures observed at $\sim1,642$ cm$^{-1}$ (19). The $\beta$-sheets are typically observed at $\sim1,636$ cm$^{-1}$ and 1,670–1,690 cm$^{-1}$. However, this type of difference spectrum may also shift due to a change in the environment of only part of the peptide backbone. So-called “doorway” shifts have also been described in KcsA K$^+$ channels (20). In this case, the difference spectra are due to different ions, which reveal small structural changes within one type of secondary structure element. Thus, the difference mode observed between which reveal small structural changes within one type of secondary structure (20). In this case, the difference spectra are due to different ions, at 1,571 cm$^{-1}$ (H/D exchange ($\nu$16)) and signals at $\nu$(C-N) moves to near 1,445 cm$^{-1}$, mixing with other modes to form a new band called amide II*. The changes in the spectra upon H/D exchange confirm that most of the signals in the difference spectra at positions lower than 1,700 cm$^{-1}$ originate from the protein backbone.

**Effect of Mutations on Glu325 SEIRAS.** To identify side chains sufficiently close to Glu325 to perturb the pKa, a systematic study on the effect of potentially important mutations on the SEIRAS difference spectrum of Glu325 was undertaken (Fig. 2 and Table 1). Each mutant was introduced into the G46W/G262W background (LacYww) to ensure high stability at alkaline pH (21). Importantly, SEIRAS data for WT LacY and LacYww, as well as the corresponding E325A mutants, are indistinguishable (12). Difference spectra between pH 7.0 and an alkaline pH at which Glu325 is essentially completely deprotonated were carried out with each mutant (Fig. 3A): (i) pseudo-WT LacYww, (ii) LacYww/E325D, (iii) LacYww/K319L, (iv) LacYww/R302K, (v) LacYww/F243W, (vi) LacYww/H322Q, and (vii) LacYww/E325A. The alkaline pH for each mutant was selected to be as close to complete deprotonation as possible. As a result, the vibrational mode of the side chain of Glu325 is observed as a negative signal close to that observed for the pseudo-WT at 1,746 cm$^{-1}$, a position that is boxed in Fig. 3A.

The difference spectrum of mutation E325D (Fig. 3A) exhibits a shift of the COOH vibrational mode to 1,728 cm$^{-1}$, a position typical of a more hydrophilic environment. The COOH in Asp or Glu at position 325 does not have the same position, and a shift of 18 cm$^{-1}$ is likely due to a change in microenvironment, hydrogen-bond strength, or a combination of both. This kind of shift was observed earlier for mutations from Glu to Asp andvice versa (22). The amide I/II region does not change significantly in difference spectra of the mutant, arguing against a change in the structural integrity of the protein. With mutations R302K and R302A, only small shifts are observed for the signal at 1,746 cm$^{-1}$. However, with mutation R302K, the difference signal is significantly broader, an effect that may be due to a higher degree of freedom of Glu325 at this position. Mutation of residue H322Q (Fig. 3A) causes a shift in the Glu325 COOH vibration to 1,737 cm$^{-1}$, which suggests stronger hydrogen bonding in the immediate environment of the residue without significant perturbation of the overall structure.

**Table 1. Overview of studied mutants**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Peak position, cm$^{-1}$</th>
<th>pKa</th>
<th>Sugar/H$^+$ symport</th>
<th>Discussed role of studied residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacY WT</td>
<td>1,747</td>
<td>10.5</td>
<td>Yes</td>
<td>H$^+$ translocation</td>
</tr>
<tr>
<td>LacYww</td>
<td>1,747</td>
<td>10.5</td>
<td>No</td>
<td>H$^+$ translocation</td>
</tr>
<tr>
<td>E325A</td>
<td>No signal</td>
<td>nd</td>
<td>No sugar/H$^+$ symport, but transmembrane sugar exchange (1, 2, 15)</td>
<td></td>
</tr>
<tr>
<td>Mutants</td>
<td>LacYww</td>
<td>E325A</td>
<td>No signal</td>
<td>nd</td>
</tr>
<tr>
<td>E325D</td>
<td>1,730</td>
<td>8.3</td>
<td>15% of WT (8, 16)</td>
<td>H$^+$ translocation</td>
</tr>
<tr>
<td>R302K</td>
<td>1,758,1,743</td>
<td>8.4</td>
<td>No</td>
<td>H$^+$ translocation</td>
</tr>
<tr>
<td>R302A</td>
<td>1,741</td>
<td>10.3</td>
<td>No sugar/H$^+$ symport, but transmembrane sugar exchange (15, 16)</td>
<td>H$^+$ translocation</td>
</tr>
<tr>
<td>H322Q</td>
<td>1,734</td>
<td>10.1</td>
<td>No</td>
<td>Ligand to sugar</td>
</tr>
<tr>
<td>Y236F</td>
<td>—</td>
<td>—</td>
<td>Decreased binding affinity and little transport</td>
<td>Hydrogen bond donor to H322</td>
</tr>
<tr>
<td>D240A</td>
<td>1,749</td>
<td>9.8</td>
<td>No</td>
<td>Salt-bridged K319/D240</td>
</tr>
<tr>
<td>F243W</td>
<td>1,740</td>
<td>10.1</td>
<td>30% of WT</td>
<td></td>
</tr>
<tr>
<td>K319L</td>
<td>1,750</td>
<td>10</td>
<td>Reduced binding</td>
<td>Salt-bridged K319/D240</td>
</tr>
<tr>
<td>L229F</td>
<td>—</td>
<td>—</td>
<td>No</td>
<td>Part of hydrophobic pocket around E325</td>
</tr>
</tbody>
</table>

nd, not determined.
In the spectrum of the K319L mutant, the signal discussed is found at 1,750 cm$^{-1}$, similar to the D240A mutation (Fig. 3A). The amide signature for these two mutants is clearly changed compared with the pseudo-WT. While there is a broad positive peak at 1,674 cm$^{-1}$ with a shoulder at 1,722 cm$^{-1}$ and a negative signal around 1,625 cm$^{-1}$ for the pseudo-WT and the mutants discussed above, the signals are inverted here, suggesting a strongly modified change in conformational reorganization that takes place upon changing pH. Residues D240 and K319 are weakly salt-bridged both structurally (Fig. 1) and functionally (23). A strong perturbation of the salt bridge in the mutant can explain the conformational changes observed. Nevertheless, the influence on the Glu325 infrared signature is negligible.

Three additional mutants have been studied that show strongly perturbed spectra (Fig. 3B). In Y236F, a signal at 1,744 cm$^{-1}$ was obtained for the step to pH 10.5, but the mutant is unstable and the amide I signature was different for each pH value studied. In L329F, no signal could be identified except for some conformational changes in the amide I/II range. Both residues are located in the hydrophobic patch surrounding Glu325 (Fig. 2). Finally, the effect of mutant Y236F was tested. Again, the signature of Glu325 was not identified, and for each pH step, the amide I signature was different, as can be seen when comparing the step to pH 10 and to pH 10.5. It is noted that Tyr236 is hydrogen-bonded to H322 (24). Mutations in the hydrophobic pocket of Glu325 perturb structure and the conformational flexibility of LacY.

**Effect of Mutations on the pK$_a$ of Glu325.** To test for an effect of each mutation on the pK$_a$ of Glu325, difference spectra were obtained for each mutant from pH 6.0–12.0. The pH-dependent increase in the negative signal at $\sim$1,745 cm$^{-1}$ was plotted as a function of pH (Fig. 3A), except for mutation E325A, where no signal is observed (6). However, mutation E325D exhibits an acidic shift in the pK$_a$ from $\sim$10.5 to $\sim$8.3 (Fig. 4), consistent with a change in the microenvironment of the COOH. Mutation E325D retains 15–20% of WT transport activity with reduced affinity (4, 8, 16). Mutant R302A is also defective with respect to affinity for galactoside (4), but catalyzes transmembrane exchange (15), and the mutation has little or no effect on the pK$_a$ of Glu325 (Fig. 3A). However, mutation R302K is particularly interesting, as it causes the pK$_a$ of Glu325 to decrease to pH 8.3 (Figs. 3C and 4B), clearly an indication of interaction between the two side chains. The Glu325 signal at 1,745 cm$^{-1}$ can be observed already at pH 8 and pH 8.5 in the difference spectra (Fig. 3C).

Unexpectedly, mutation H322Q, which is only one helix turn from Glu325, has little or no effect on the pK$_a$ for Glu325 (Fig. 4). Since His322 is liganded to the galactopyranosyl moiety of LacY substrates, the binding affinity of the mutant and all transport reactions are strongly diminished (4). Mutations D240A and K319L disrupt a relatively weak salt bridge (23) and cause very small, but reproducible, acid shifts in the Glu325 titration. Finally, mutations Y236F (helix VII) and L329F (helix X) yield unstable Glu325 pH titrations as mentioned above, and the spectra differ at each pH tested. Both of

![Fig. 1. Cytoplasmic view of the E325 environment and of the positions of the mutations (Protein Data Bank ID code 2V8N).](image1)

![Fig. 2. Hydrophobic pocket around Glu325 (Protein Data Bank ID code 2V8N).](image2)
these side chains comprise part of the hydrophobic pocket in which Glu325 is situated.

Discussion

LacY drives accumulation of lactose against a 50- to 100-fold concentration gradient (25), with a turnover number of ∼20 s⁻¹. Both ΔΨ (interior negative) and ΔpH (interior alkaline) have quantitatively the same effect on transport with a 50- to 100-fold decrease in Kₘ and the same thermodynamic equilibrium. In opposition to time-honored conjecture, the Kₐ for galactosides on either side of the membrane is essentially the same in the absence or presence of Δ~μH⁺ (26). In the absence of Δ~μH⁺, there is three- to fourfold inhibition of lactose transport in the presence of deuterium oxide (D₂O), indicating that deprotonation is rate-limiting (7, 27–29). However, in the presence of Δ~μH⁺, there is a driving force on the H⁺, deprotonation is no longer rate-limiting, and the transport rate is unaffected by D₂O. It is particularly noteworthy that a number of experimental findings indicate strongly that Δ~μH⁺ functions kinetically as a driving force on the H⁺, while alternating access is driven by sugar binding and dissociation (30).

Clearly, a major problem for LacY turnover is deprotonation when the midpoint for both galactoside and H⁺ binding is pH 10.5, which translates into a Kₐ for H⁺ of ∼30 pM. As discussed, it is unlikely that either decreasing the cytosolic H⁺ concentration or the pKa of Glu325 is a realistic possibility. An increase in water accessibility to the hydrophobic site in which Glu325 is located is a possibility. However, as shown by three independent H/D exchange studies (31–33), the backbone amide protons in LacY are largely accessible to water. Another possibility is that Arg302 and Glu325 may be in closer proximity in another conformation of LacY (13, 14), and there is experimental evidence that distance between the two residues is important in this regard (16). The data obtained here indicate that Arg302 interacts with Glu325. While the R302A mutation does not significantly change the microenvironment of Glu325, introduction of a Lys induces a downshift of approximately two pH units and a broader COOH vibrational mode characteristic of a residue with significant rotational freedom. Although Arg and Lys
are both positively charged, the distribution of the charge is different, and the delocalized charge on Arg is apparently critical for the $pK_a$ value measured here. However, neither the R302K mutation nor the R302A mutation causes gross alteration in structure, as is also the case for other mutations in the vicinity of Glu325 that disrupt a salt bridge or cause a change in conformation, but do not affect the $pK_a$ of Glu325.

**Materials and Methods**

**Construction of Mutants, Purification of LacY, and Reconstitution.** Construction of mutants, expression in *E. coli*, and purification of LacY were performed as described before (4, 6). All constructs contained a C-terminal His6 tag that was used for affinity purification with Talon resin. Purified proteins (10–15 mg/mL) in 50 mM sodium phosphate [NaPi/0.02% DDM (pH 7.5)] were frozen in liquid nitrogen and stored at −80 °C until use.

**Surface Modification of the Silicon Crystal and Protein Immobilization.** On the surface of a Si attenuated total reflection (ATR) crystal, a thin gold layer was formed by chemical deposition as described previously (6). The crystal was dried under an argon stream, and 40% (wt/vol) NH₄F was added for 1 min to remove the Si 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 (vol/vol/vol) of 15 mM NaAuCl₄ + 150 mM Na₂SO₃ + 50 mM Na₂SiO₃ + 50 mM Na₂H₂O. After reaching the temperature, the prism was covered with the solution for 40 s, and washed with water, followed by drying with a stream of argon.

After formation of a gold layer on the Si crystal, a nickel-nitritolriatic acid self-assembled monolayer was adapted from techniques reported by Grytsyk et al. (6), Ataka and Heberle (34), and Kriegel et al. (35). First, 1 mg/mL 3,3'-dithiodipropionic acid di(N-hydroxysuccinimide) ester (DTSP) in dimethyl sulfoxide (DMSO) was allowed to self-assemble for 1 h. After monolayer formation, excess DTSP was washed away with DMSO and the crystal was dried under an argon stream. Afterward, the self-assembled monolayer was immersed in 100 mM Na₂,N₂-bis(carboxymethyl)-L-lysine in 0.5 M K₂CO₃ at pH 9.8 for 3 h and rinsed with water. Finally, the surface was incubated in 50 mM Ni(CIO₄)₂ for 1 h.

**Infrared Spectroscopy.** A configuration allowing the simultaneous acquisition of Fourier transform infrared (FTIR) spectra in the ATR mode with perfusion of solutions with well-defined composition was employed, including a silicon crystal with a 3-mm surface diameter as a multireflection ATR unit. All experiments were carried out with a Bruker Vertex 70 FTIR spectrometer (Globar source, KBr Beamsplitter, LN-MCT detector) at a scanner velocity of 40 kHz. The measurements were carried out at ~7 °C. Solutions were kept in ice before use. For the data presented here, the pump speed was kept constant at a flow rate of 0.2 mL min⁻¹. Before each perfusion step, the input tube was carefully washed with water and buffer.

**Difference Spectra.** To monitor pH-induced difference spectra, one perfusion buffer with a constant pH value of 7.0 was used as a reference (25 mM KPi/100 mM KCi/0.01% dodecyl-D-maltopyranoside) and a second perfusion solution with the same composition, but at different pH values ranging from 8.0 to 11.5, was employed. After equilibration for 30 min with the KPi buffer (pH 7.0) a reference spectrum was recorded as background and the perfusion solution was changed to the second solution (pH range: 8.0–11.5). After 20 min (pH 8.0–11.5) minus pH 7.0 difference spectra were recorded. The new state of the protein was recorded as background, and the solution was changed to pH 7.0. Finally, the pH 7.0 minus (pH 8.0–11.5) difference spectra were obtained. The same procedure was repeated at least five times, and the difference spectra were averaged. Baseline correction was done wherever necessary.

**Difference Spectra of LacY After H/D Exchange.** The perfusion solution was prepared in D₂O for the analogous pD values as described above. After perfusing with the pD 7 solution, the completed exchange was confirmed on the basis of the typical shift of the amide II band. The experiments were then performed like those carried out in H₂O.

**ACKNOWLEDGMENTS.** We thank Aurore Pereau (University of Strasbourg) for performing control experiments on mutant Y236F during her master internship. H.R.K. acknowledges the University of California, Los Angeles and a gift from Ruth and Bucky Stein. P.H. acknowledges the University of Strasbourg, Institute of Advanced Studies; the International Center Foundation Research in Chemistry (icFRC); and the CNRS and Initiative d’Excellence (IDEX) of the University of Strasbourg for financial support. The work was also supported by Nih Grant GM120043 and National Science Foundation Eager Grant MCB1747705 (to H.R.K.).

---

**Fig. 4.** Titration curves for the ν(C = O) vibrational mode of Glu325 at 1,746 cm⁻¹: pseudo- WT with (red) and LacYww/E325D (blue) (A); LacYww/R302K (purple) and LacYww/R302A (green) (B); and LacYww/D240A (orange), LacYww/K319L (cyan), and LacYww/H322Q (gray) (C).